Effects of environmental toxicants in Atlantic cod (*Gadus morhua*) from the Inner Oslofjord

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Master thesis in Ecotoxicology
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Abstract

Cod is an important species in Norway economically, ecologically and as a food source. Atlantic cod in the inner Oslofjord is exposed to a range of environmental toxicants. It is therefore important to monitor the health of the cod. In this thesis the effects of environmental toxicants on cod in the inner Oslofjord was investigated. This was done by measuring physiological indices and by applying biomarkers at different organisational levels. Cod was sampled from the inner Oslofjord with the outer Oslofjord used as a reference site. Based on liver somatic index and condition factor, cod from the inner and outer Oslofjord seemed to be in similar overall condition. Gonadal somatic index showed that cod from the inner fjord had matured earlier than cod from outer. This indicates either different reproductive patterns for the two populations or exposure to estrogens for cod in the inner Oslofjord. Biomarkers revealed increased exposure to PAHs in the inner Oslofjord. Effects from this exposure, and other toxicants, were revealed by biomarkers for EROD and CYP1A. CYP1A expression was up-regulated while EROD activity may have been inhibited. This implies exposure to toxicants such as TBT. No significant differences in exposure to metals between the two populations were found measured by MT and ALA-D. Differences in GSI and Vtg expression between the two populations were found, indicating exposure to estrogens for the inner Oslofjord cod. AChE activity was found to be inhibited in outer Oslofjord cod, similar to previous years, showing that cod is still exposed to AChE inhibiting substances.

Abbreviations

AChE	Acethylcholin esterase
ACTB	β-actin
Ah-receptor	Aryl hydrocarbon receptor
ALA-D	δ-aminolevulinaciddehydrogenase
ANOVA	Analysis of variance
ATC	Acethylthiocholine iodide
B(a)P	Benzo(a)pyrene
BSA	Bovine serum albumin
cDNA	Complementary DNA
CF	Condition factor
Co(NH3)6Cl3	Hexaamminecobalt(III) chloride
Cp	Crossing point
CYP	Cytochrome P450–dependent monooxygenase
CYP1a	Cytochrome P4501A
DDT	Dichlorodiphenyltrichloroethane
DMAB	p-dimethylaminobenzaldehyde
DMSO	Dimethyl sulfoxide
DOC	Dissolved organic carbon
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DTT	Dithiothreitol
EDTA	Ethylendiamintetraacid
EF1A	Elongation Factor 1-Alpha
EROD	7-ethoxy-resorufin-*O*-deethylase
Exp.factor	Expression factor
GSI	Gonadal somatic index
GST	Glutathione *S*-transferase
HgCl2	Mercuric chloride
HO-1	Heme-oxygenase-1
HPLC	High-performance liquid chromatography
hsp70	Heat shock protein 70
HSPs	Heat-shock-protein
KCl	Potassim chloride
LSI	Liver somatic index
mRNA	Messenger mRNA
MT	Metallothionein
NaCl	Natrium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NEC	No enzyme control
NH4OH	Ammonium hydroxide
NIVA	Norwegian Institue for Water Research
NTC	No template control
OCP	Organochlorine pesticides
3-OH-B(a)P	3-Hydroxy-benzo(a)pyrene

OH-phyrene	Hydroxy-phenanthrene
OH-pyrene	Hydroxy-pyrene
PAH	Polycyclic aromatic hydrocarbons
PBG	Porphobilinogen
PCB	Polychlorinated biphenyls
RT-qPCR	Real time quantitative polymerase chain reaction
TBTs	Tinorganic compounds
TCA	Trichloroacetic acid
UGT	Uridine diphosphate glucuronosyltransferases
Vtg	Vitellogenin

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1 Introduction

1.1 General background

The environment receives foreign contaminants such as polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs) and heavy metals (Van Der Oost et al., 2003). Many such contaminants will eventually end up in the aquatic environment, either due to direct release or to hydrological and atmospheric processes (Stegeman et al., 1994). The main sources of these pollutants are natural oil leaks from the sea floor, runoff from land, offshore installations, shipping and deposition from air and transport by water currents (Macdonald et al., 1996). Several studies have investigated exposure and impact from these compounds on marine fish species, ranging from different physiological changes, chemical measurements of their metabolites and/or effects measured at different sub-cellular levels e.g. enzyme activity or gene expression (Abou-Donia et al., 1967; Jackim, 1973; Stegeman et al., 1980; Christensen et al., 1982; Bonham et al., 1984; Kleinow et al., 1987; Olsson et al., 1990). Monitoring based on such measurements is important in order to evaluate the overall effects in fish caused by exposure to toxicants and time series can also provide important information about temporal trends (Morrisey, 1993; Nicholson et al., 1997).

The inner Oslofjord, covering approximately 193 km^2, is surrounded by the area in Norway that is most heavily populated (Breivik et al., 2004). The inner and outer Oslofjord are separated by the Drøbak sound, with a comparatively shallow sill of 19.5 meter which is responsible for a low exchange of water masses (Berge et al., 1987). Previous studies have reported increased primary production and euthrophication in the inner Oslofjord. This might cause increased concentrations of dissolved organic carbon (DOC) in the marine ecosystem, which may further affect the behaviour of pollutants (Abdullah et al., 1982; Ding et al., 1997). The increased DOC may also cause anoxic condition in bottom waters and oxygen deficiency has been seen to affect the uptake of toxicants in fish. This is because the flow rate of water through the gills increases and this can lead to enhanced absorption of toxicants (Porte et al., 2002). Higher pollutant concentrations are also usually found in the sediment compared with the water column, since lipophilic toxicants tends to bind to sediment particles (Richards et al., 1986). The inner Oslofjord is topologically restricted with two deep basins; Vestfjord and Bunnefjord and three more shallow basins; Lysakerfjord, the harbour area of Oslo, and the

Bækkelaget basin. These restrictions can consequently reduce the transport of pollutants out of the fjord and into the North Sea and Skagerrak (Arnesen, 2001; Skei, 2009). These properties combined with runoff from industrial and other anthropogenic activities may cause environmental pollutants to be trapped within the fjord (Skei, 2009). Organochlorines have for example generally been suggested to have a slower clearance rate in fjords than in rivers and lakes (Berg et al., 1998).

In monitoring purposes it is considerably more biologically relevant to use indicator species for specifying the effects caused by environmental pollution compared with using physical or chemical measurements to reveal the concentration in the biota (Phillips, 1977). Atlantic cod (*Gadus morhua*) is often used in monitoring purposes since it is widely distributed, has a high abundance, and is known to accumulate environmental pollutants. It is also considered being relatively stationary (Hop et al., 1992). Cod has a long tradition within risk assessment and for food consummation. It is used as a key species in monitoring programs such as the Co-ordinated Environmental Monitoring Programme (Green et al., 2010a). It is also an important species along the Norwegian coast, both commercially and ecologically. Cod larvae drift with ocean currents, and this causes gene flow between cod populations (Stenseth et al., 2006). Despite this gene flow, significant genetic differentiations have been observed between populations. Cod from the inner Oslofjord is therefore suggested to be genetically different from cod in the outer Oslofjord (Knutsen et al., 2003). However the literature reveals uncertainties in the genetic variation between populations of cod (e.g. Imsland et al., 2002). The feeding behaviour of cod changes with body size. Individuals larger than approximately 50 cm in length have been seen to base their diet mainly on other fish, but also on sediment dwelling species such as isopods, amphipods, and polychaetes (Hop et al., 1992). Since many environmental toxicants are known to biomagnify in the food web, the feeding behaviour of fish can therefore affect their overall exposure to pollution (Van Der Oost et al., 2003). Cod mainly feed in deep waters which has been observed to influence their uptake of environmental pollution in the inner Oslofjord (Schlabach et al., 2007). The spawning period of cod is between late winter to spring, but local differences have been observed (Brander, 1994; Wieland et al., 2000).

1.2 Pollution status of the inner Oslofjord

1.2.1 Polycyclic aromatic hydrocarbons

The national release of PAHs has been reduced by approximately 42% from 1995 until 2008 (Sørensen, 2011) and measurements of PAH content in mussels revealed a downward trend of PAH concentration between 2006 and 2009 in the inner Oslofjord (Berge, 2011). Remarkably however, PAH metabolite concentrations in bile from cod from the inner Oslofjord have increased between 1984–2006 (Green et al., 2010b). Other measurements of PAH metabolites in inner Oslofjord cod bile conducted in 2002, revealed higher concentrations of hydroxyl-pyrene (OH-pyrene) and hydroxyl-phenanthrene (OH-phenanthrene) in comparison with the outer fjord (Holth, 2004) while there in 2008 were seen higher concentrations of OH-pyrene in the inner Oslofjord (Imrik, 2010). These findings might indicate that the exposure to pyrene in cod still were higher in the inner Oslofjord compared with the outer Oslofjord. Despite the national reduction in PAH release, other sources can contribute to the possible higher amount in the inner fjord. There have been estimated to be transported 20–60 tons PAHs by air per year into Norway and an annual leakage of 23 tons creosote contaminated soil (Sørensen, 2011).

1.2.2 Metals

Cod collected in the inner Oslofjord between 1984 and 2009 have been observed to contain elevated levels of mercury (Hg) in muscle (Green et al., 2010b). The same tendencies have also been found in freshwater fish species, with a substantial increase of Hg in recent years (Fjeld et al., 2009). In contrast, national release of this metal into the environment is considered to have been reduced with 60% from 1995 to 2005, most likely as a consequence of the prohibition of mercury addition in products in 2008 (Green et al., 2010b). Approximately 20 tons of mercury polluted soil in Norway is considered to leak out every year, and in 2008 2.2 tons were in addition calculated to be brought in by atmospheric transport (Sørensen, 2011). These sources may contribute to increased Hg concentrations in the inner and outer Oslofjord, but other abiotic factors have been suggested to cause the elevated Hg levels seen in freshwater fish like increased temperature and DOC content (Fjeld et al., 2009).

Cadmium (Cd) is another metal of concern that is found in the inner Oslofjord. Elevated Cd content has been observed in cod liver in recent years (Green et al., 2010b). Cd-containing waste effluent has been reduced by 70% nationally, but still approximately 1.5 tons of cadmium were released from polluted soil in 2008 (Sørensen, 2011). Cd is also thought to be transported into the inner Oslofjord via ocean currents (Green et al., 2010a).

The lead discharge were found to be reduced by 70% from from 1995 to 2008 (Sørensen, 2011) and mussels measured between 1984 and 2009 were not polluted with mercury. Cod from inner Oslofjord is also thought to be less affected by lead in recent years (Green et al., 2010b).

1.2.3 Toxicants inhibiting acetylcholine esterase activity

Previous monitoring studies have demonstrated that cod from the outer Oslofjord was exposed to higher concentrations of toxicants which inhibit acetylcholine esterase (AChE) activity compared to cod from the inner Oslofjord (Imrik, 2010). Many substances have been seen to inhibit the enzyme activity such as the pesticides carbamates and organophosphates, but also heavy metals (Olson et al., 1980). Since it is unclear which toxicant is causing the inhibition of AChE, it is difficult to discuss the status of these toxicants in the inner and outer Oslofjord. Since pesticides is one toxicant group known to inhibit AChE (Payne et al., 1996) one must consider the sources of this group. The total sale of pesticides decreased from 1529 to 530 tons in Norway between 1985 and 2005 (Snellingen-Bye et al., 2006). Approximately all pesticides used in Norway, and/or their metabolites, have been found in the marine environment (Ludvigsen et al., 2005). A recent study have reported that for many pesticides detected in European waters, the levels were above predicted effect concentrations (Von Der Ohe et al., 2011). Formulating plants and manufacturing processes are also thought to contribute to the release of pesticides (Parveen et al., 2005).

1.2.4 Other environmental toxicants of concern

There has been observed elevated levels of exposure to endocrine disruptors in male cod from the inner Oslofjord (Scott et al., 2006). More than 50 substances have been suggested to be estrogens (Rolland, 2000) and the presence of complex mixtures of chemicals in the environment makes it difficult to assess which one is affecting endocrine processes (Petersen et al., 2011). Sources of estrogen compounds includes tannery effluents, paper and pulp mill

effluents, crude oil derivatives, sewage effluents and discharge from wastewater treatment plants (García-Reyero et al., 2004; Thorpe et al., 2003) and one of the most potent estrogens found in wastewater is the birth control rethinylestradiol (Thorpe et al., 2003). Studies have indicated that even extremely low concentrations (a few nanograms per liter) of estrogens can cause feminisation of fish if they are exposed during early development (Metcalfe et al., 2001). There have also been found high levels of the anti-estrogenic group tinorganic compounds (TBTs) around marinas in the inner Oslofjord. This was unexpected due to the ban of organic tin compounds used as anti-fouling paint on boats since 2003 (NGI, 2010). The substances have however high persistence in the environment and also sea currents are known to contribute with TBT input into the Oslofjord (Green et al., 2010a).

Also other toxicants are found in the inner Oslofjord such as PCBs (Green et al., 2010b). Although there has been reported a national reduction of almost 70% of PCBs from 1995 to 2008, 70 tons of PCB polluted ground is still believed to exist in Norway causing substantial annual leakage (Sørensen, 2011). Dioxins are another toxicant group present in the Oslofjord and their release have been reduced with almost 70% in the same time period as above and only a small proportion of this release ends up in the sea (Sørensen, 2011).

1.3 Detecting effects of environmental toxicants

1.3.1 Physiological indices

Age and sex are important physiological factors known to influence the effects of environmental pollutants (Kleinow et al., 1987). Seasonal patterns of nutrient storage and depletion can be monitored in cod by measuring changes in weight of compartments like carcass, liver, and gonads (Schwalme et al., 1999). Excess energy from basal maintenance will be available for either growth or reproduction and can be measured by increased mass in one of the compartments mentioned above (Ewald, 1995). The Fulton condition factor K, represents the mass of an individual relative to its body length which reflects the fat content and the overall energy status of individual fish (Schmidt-Nielsen, 1984). Energy reserves in Atlantic cod are known to change seasonally with a maximum during fall and a minimum during spring in relation to spawning (Dutil et al., 2000). The condition factor is also considered to be influenced by several other factors than nutritional status, like pathogen load and chemical exposure. This can result in both elevated and reduced values, but traditionally a

low factor is considered to indicate decrease in energy reserves (Azmat et al., 2007; Anderson et al., 1996).

The liver somatic index (LSI) reflects the lipid content of the liver which is considered to be a direct measure of food availability and fitness (Adams et al., 2011). The liver is the primary site of metabolism for most chemicals and therefore often the first organ to be exposed for metabolites (Ewald, 1995). Increased liver size as a result of induction or activation of biotransformation enzymes, or reduced size due to injury and death of hepatic cells has been described (Mdegela et al., 2010). The usefulness of condition factors such as LSI in relation to environmental contaminant exposure like PCBs, OCPs and PAHs has been shown in several studies (reviewed in Van Der Oost et al., 2003).

Cod mainly accumulate lipids in the liver and this energy is transferred to the gonads when vitellogenesis occurs. As a consequence of this, negative correlation between increased gonad weight and liver weight in fish have been observed (Lambert et al., 1997). The gonadal somatic index (GSI) measures the weight of the gonads in relation to the somatic weight and may indicate the gonad maturity of the fish (Gunderson et al., 1988). Studies have also shown that GSI levels, as well as testis growth, may be affected by estrogens (Jobling et al., 1996; Noaksson et al., 2001). The energy used to metabolise toxicants are also thought to affect the energy available for gonadal investments in fish. Therefore fish with good overall condition may be less affected by pollutants.

1.3.2 The use of biomarkers for monitoring purposes

The term “biomarker” describes biological endpoints used to uncover effects of environmental toxicants on organisms (IPCS, 1993). Biomarkers can be used to indirectly measure exposure to toxicants such as metabolite content, and also the eventual effects caused by this exposure (Timbrell, 1998). There are several advantages of using biomarkers such as high sensitivity and that the response caused by toxic exposure measured by using biomarkers is considered to be absolute and can therefore be applied to environmental management (Ewald, 1995). When applying biomarkers as a monitoring tool, there are several considerations to make. In a natural population there will always be high degrees of inter-individual variations in response of exposure to toxicants (Gil et al., 2001). It is important to include the total variation in response from all individuals as this can provide important information about ecological changes induced by pollution (Depledge, 1990). There are also many environmental and ecological factors that can influence the response of organisms to

particular stressors. One should therefore be careful with extrapolating responses seen by using biomarkers only, to possible effects on populations (Adams et al., 1999). But relationships between concentration of toxicants in tissue, response in biomarkers, and physiological status have been found, e.g. in Atlantic cod (Hylland et al., 2009). The time aspect is another important consideration when monitoring effects of toxicants on organisms. Chronic exposure to low levels of contaminants does not necessarily result in similar effects as after acute exposure. Multigenerational effects have also been demonstrated on fish exposed to toxicants, where no adverse effects were seen in the exposed individual, but only in their offspring (Rolland, 2000; Schwaiger et al., 2002; Ewald, 1995).

The use of biomarkers as "early warning signals" is based on a theoretical time-dependent relationship of responses from low to high levels of biological organisation (Holth et al., 2008). It is generally assumed that effects from pollution will initially be present at the cellular level, then later in tissues followed by possible physiological effects. Different biomarkers are used to measure effects at different levels of organisation (Lam et al., 2003). The main advantage of using biomarkers at low levels of biological organisation is the possibility to detect deleterious effects from contaminant exposure at an early stage (Underwood et al., 1988). On the other hand, higher organisation levels are more biologically relevant. Fewer precautions also need to be included when trying to extrapolate to individual or population level.

When using biochemical biomarkers, measurements of enzyme activity are useful due to their high degree of specificity and rapid response to changes in the surrounding environment (Marco et al., 1996). However, the specificity of the biomarkers at enzyme level varies. Inhibition of e.g. δ-aminolevulinic acid dehydratase (ALA-D) activity is considered being a specific biomarker for lead exposure (Hodson, 1977) while ethoxyresorufin-*O*-deethylase (EROD) activity for instance, can be affected by several internal and external factors that is not only due to toxicants (Whyte et al., 2000).

Studies have indicated that biomarkers based on gene expression are more sensitive and can detect effects from toxicants exposure even when effects are not seen at a protein level (Denslow et al., 2001; Reid et al., 2003). Full or partial characterization of the transcriptome for some fish species, such as the Atlantic cod, has made it possible to use expression of mRNA as a tool to identify chemical exposure (Mcclain et al., 2003). There are several advantages of mRNA-based biomarkers over protein measurements. For instance that they are considered to have a more rapid response to exposure of toxicants, that several

samples and genes can be quantified in a relatively short time, and that these biomarkers can easily be extrapolated from one species to another by designing appropriate primers (García-Reyero et al., 2004; Lattier et al., 2001). The response however, will only be measurable for a shorter time period than responses measured at a higher organisation.

1.3.3 PAHs

Measurements of PAH metabolites in fish bile is a commonly used biomarker for exposure to PAH compounds (Grung et al., 2009; Ariese et al., 2004). PAHs often tend to be introduced into the marine environment in a mixture and thereby causing mixture toxicity (Barron et al., 2004). Many toxicants in this group are known to be carcinogenic by forming DNA adducts in fish (Myers et al., 1998) which has for example been observed in Atlantic cod (Aas et al., 2000). PAHs are effectively metabolised in fish and the bile is the dominant excretion route (Kleinow et al., 1987). Hydroxy-phenanthrene (OH-phenantrene), hydroxy-pyrene (OH-pyrene) and 3-hydroxy-benzoapyrene (3-OH-BaP) are metabolites often analysed for in fish bile due to their toxicant relevancy and expected detectable concentration (Ariese et al., 2005). Small two-and three-ringed PAH metabolites (e.g. OH–phenanthrene) often results from exposure to PAHs from oil spills (Krahn et al., 1986). For the larger PAH metabolites like OH–pyrene and 3-OH–BaP, containing four and five rings respectively, incineration processes are often the main source (Ariese et al., 2004; Blahova et al., 2008). However, due to the rapid metabolism of many PAHs in fish, the concentrations of their metabolites in bile may cause underestimation of the exposure (Whyte et al., 2000).

EROD activity is a commonly used biomarker for studying the effects of exposure to PAHs. It is a sensitive measurement for determining the catalytic response of the cytochrome P450 (CYP) system in fish (Goksøyr et al., 1992; Van Der Oost et al., 2003; Elcombe et al., 1979). The activity can be measured by following the increase in fluorescence of the reaction product resorufin, since the enzyme converts ethoxyresorufin to resorufin (Burke et al., 1974). EROD activity has been observed to increase when fish are exposed to PAHs, polychlorinated naphthalenes, PCBs or dioxins (reviewed in Whyte et al., 2000). The effect of e.g. PAH exposure can also be measured by using cytochrome P450 1a (CYP1a) induction as a biomarker (Kloepper-Sams et al., 1989; Haasch et al., 1989). Fish exposed to PAHs after oil spills have been observed to show elevated expression of CYP1a (Stagg et al., 2000). Although alteration of CYP1a gene transcription is considered a sensitive indicator for exposure to PAHs (Levine et al., 1999), this biomarker can also serve as an indicator of an

organisms total toxic burden since several groups of toxicants are known to induce the CYP1a synthesis (Ewald, 1995).

Uridine diphosphate glycosyltransferase (UGT) and glutathione *S*-transferase (GST) are also gene families known to be affected by exposure to organic contaminants such as PAHs (Van Der Oost et al., 2003). These enzymes play a significant role in detoxification of foreign compounds and the syntheses of these enzymes are regulated through the same receptors as CYP1a, the aryl hydrocarbon receptors (Ah-receptors) (Nelson et al., 1993; Bradshaw et al., 2002).

1.3.4 Metals

Increased metallothionein (MT) content in tissue is widely used as a biomarker to monitor the exposure of heavy metals (e.g. Olsson et al., 1996; Hamilton et al., 1986; Roesijadi et al., 1994). The protein is synthesised in various organs but the capacity for MT induction in fish are highest in liver, small intestine and gills (Bae et al., 2005). In addition to binding non-essential metals to avoid toxic interactions with target tissues or organs, MT also binds essential metals for uptake in cells (Thornalley et al., 1985). The synthesis of MT is induced by exposure to different heavy metals like cadmium (Bonham et al., 1984; Stephen, 1989; Hogstrand et al., 1990), copper (Ahmad et al., 2000; Mccarter et al., 1983) and mercury (Sinaie et al., 2010; Cosson, 1994a). Mercury has been suggested to be the most potent metal to induce MT synthesis, followed by cadmium, silver and zinc (Cosson, 1994b). For the protein to be synthesised and the enhanced levels to be maintained, the fish must be exposed to substantial levels of metals. This level will return back to normal after the exposure (Hylland et al., 1992) which implies that any effect on MT content reflects recent exposure. Changes in MT mRNA expression is also considered being a specific indicator of metal exposure for the same reasons as for the protein, although considered to be more sensitive (Van Cleef-Toedt et al., 2001; Lie et al., 2009a). Several other factors than metal exposure can impact the synthesis of MT. In female fish, zinc is bound to MT prior to spawning and released at the start of the maturity process (Olsson et al., 1989). Hence, the MT levels drop during spawning as the zinc is no longer bound to MT proteins (Olsson et al., 1990; Hylland et al., 1992). Physiological factors like higher age and larger size have also been seen to increase the metal uptake in fish due to e.g. increased dietary intake and this can thereby affect the MT levels (Zhang et al., 2007; Dallinger et al., 1987).

δ-aminolevulinic acid (ALA-D) activity is a commonly used biomarker for lead (Pb) exposure in fish (Hodson, 1977; Burden et al., 1998; Jackim, 1973). ALA-D catalyses the synthesis of porphobilinogen (PBG), a reaction known to be inhibited by lead. By measuring the amount of PBG one can thereby estimate the enzyme activity (Hodson, 1977; Johansson-Sjöbeck et al., 1979). Although ALA-D is considered to be inhibited by lead, it has not been clarified if other metals have similar inhibition characteristics on ALA-D (Hylland et al., 2009).

MT is also, in addition to binding metals, thought to protect the cells from damage by free radicals (Olsson et al., 1995) as metals are known to cause formation of reactive oxygene species (Stohs et al., 1995). Studies have also shown that when cell cultures of fish were exposed to hydrogen peroxide, the MT synthesis increased (Kling et al., 1996). Heme-oxygenase-1 (HO-1) is involved in the cellular defence against oxidative stress, which is believed to be an important defence system in fish similar as for mammals (Applegate et al., 1991; Elcombe et al., 1979). Both MT and HO-1 are genes considered to code for stress proteins in fish (Lawrence et al., 2003).

1.3.5 Toxicants inhibiting AChE

Acetyl cholinesterase (AChE) inhibition is one of the earliest used environmental biomarkers. It is used to indentify exposure to pesticides such as organophosphorus compounds and carbamates in a dose-dependent manner (e.g. Payne et al., 1996). But also a range of other toxicants is known to inhibit AChE, such as metals (Olson et al., 1980). AChE is involved in neurotransmission and brain and muscle tissues are therefore considered suitable sites for these measurements, due to the high content of neurons (Fukuto, 1971).

1.3.6 Alteration in gene expression

Vitellogenin (Vtg) is a yolk protein produced in female fish, and the induction of this protein in males or immature fish is a well established biomarker for the presence of endocrine-disrupting chemicals in the environment (Folmar et al., 2001; Rolland, 2000). Several studies have shown up-regulation of Vtg mRNA in male individuals exposed to compounds with believed and known endocrine impact (Mellanen et al., 1999; García-Reyero et al., 2004). Unseasonally elevated levels of Vtg in female fish is also used as an indicator for exposure to such toxicants (Kleinkauf, 2004). TBT is also a toxicant known to e.g. disrupt the endocrine

system in fish by functioning as an anti-estrogene and thereby causing masculinisation of female fish (Bortone et al., 1994) and this might also cause e.g. alteration in Vtg expression.

The induction of heat-shock proteins (HSPs), also called stress proteins, has been suggested to be valuable biomarkers for exposure to contaminants and other stressors like heat -stress, and heat-shock protein70 (hsp70) is one of them (Sanders, 1994). Increased hsp70 expression has for example been observed in rainbow trout exposed to metals (Williams et al., 1996).

1.4 Aims and hypotheses

Anthropogenic activity causes input of environmental pollutants into the marine environment. There is a range of biomarkers available to investigate potential impacts of pollution at different levels in an organism. Biomarkers facilitate the detection of effects of toxicants at an early stage before they may progress. Originally, biomarkers have measured physiological, chemical and biochemical effects, but gene expression analyses also have the potential to be used as sensitive biomarkers. Cod was chosen as indicator species because of its economic importance, its long tradition as a food source in Norway and the fact that it has been used as an indicator species for over 20 years. Cod is therefore widely used for monitoring purposes, including regular studies in the inner Oslofjord. When employing biomarkers to reveal effects from toxicant exposure on an indicator species from one specific area, one must compare against a reference site that is considered clean. Since organisms are often exposed to contaminant mixtures and because many variables are known been to impact the severity of toxicant exposure, it is important to clarify any relationships between the different parameters and biomarker responses. The overall aim of this thesis was therefore to examine effects of environmental toxicants on cod from the inner Oslofjord.

The main aim was further divided into the following hypotheses:

H_0: *There were no significant differences in physiological indices between cod from the inner and outer Oslofjord or between females and males.*

H_0: *There were no significant differences in the amount of PAH metabolites or response to PAH exposure in cod between the inner and outer Oslofjord or between females and males.*

H_0: *There were no significant differences in response to metals in cod between the inner and outer Oslofjord or between females and males.*

H_0: *There were no significant differences in AChE activity in cod between the inner and outer Oslofjord or between females and males.*

H_0: *There were no significant differences in gene expression of selected genes between cod from the inner and outer Oslofjord or between females and males.*

H_0: *There were no significant correlations for the measured variables in cod from the inner and the outer Oslofjord or for females and males.*

2 Materials and methods

2.1 Trawling

Cod was sampled 23-26 September 2009 with the research vessel FF Trygve Braarud. In total, 80 individuals were sampled by trawling. 40 individuals were sampled from the inner and outer Oslofjord, respectively (Table 2.1). The trawling speed was 1.3-1.6 knots. wling areas were similar to earlier years; “Midtmeie” close to Steilene in the inner Oslofjord (Fig. 2.1) and “Travbanen” in the outer Oslofjord (Fig. 2.2). The cod were sampled in November to minimise the influence of hormone levels related to maturation. To standardise the selection of fish, only individuals of approximately the same size were sampled in addition to avoiding diseased individuals. Following trawl retrieval, cod were quickly transferred to flow-through seawater tanks on deck where they were kept alive until sampling (never longer than four hours).

Table 2.1: Number of individual cod sampled from each area.

Area	Inner Oslofjord	Outer Oslofjord
Females	21	24
Males	19	16

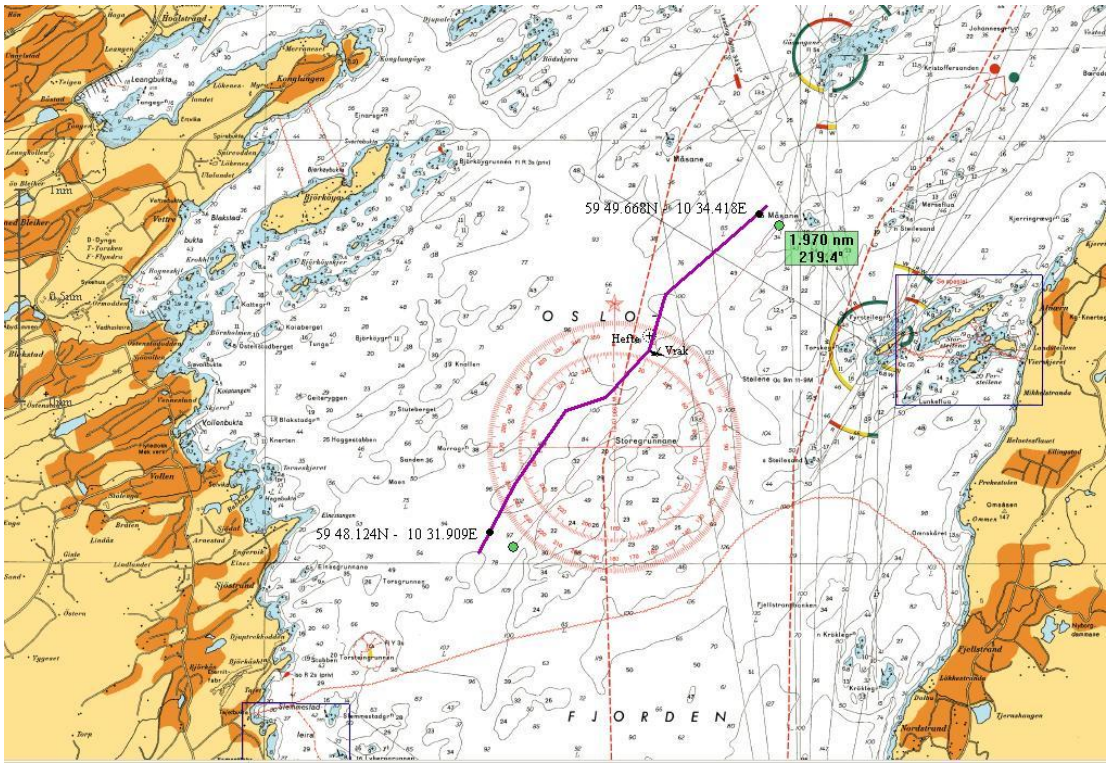


Figure 2.1: The inner Oslofjord with trawling track indicated (purple lines).

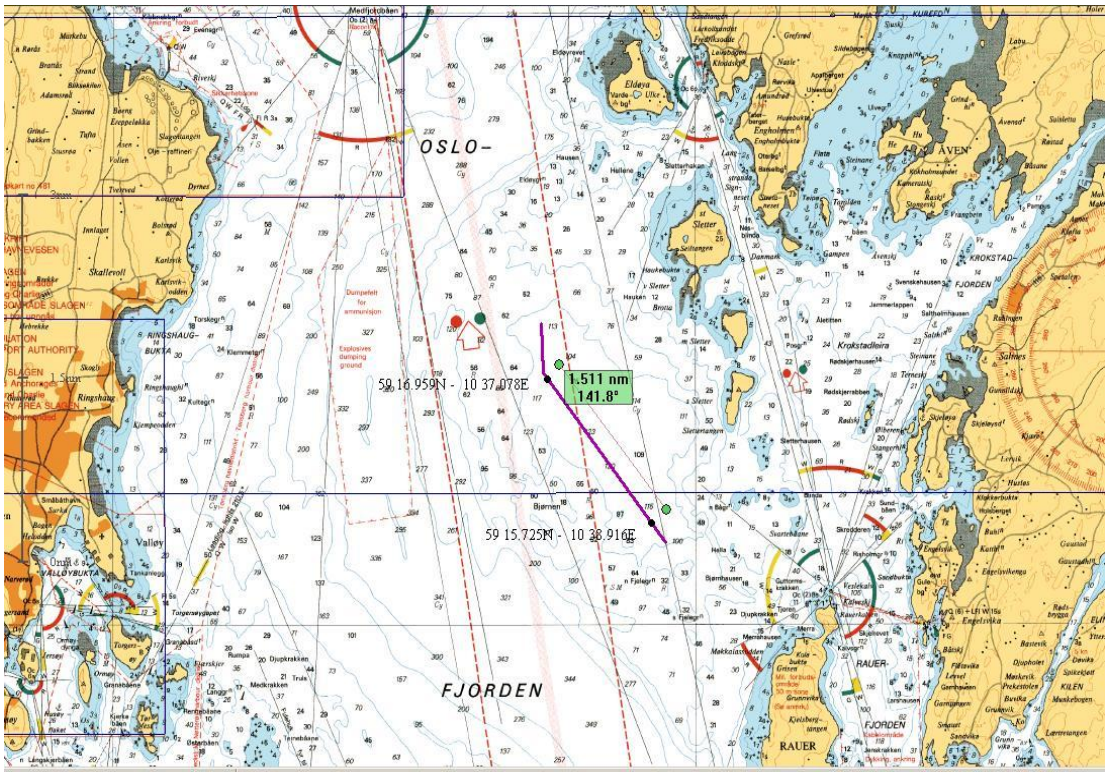


Figure 2.2: The outer Oslofjord with trawling track indicated (purple lines).

2.2 Sampling

Prior to sampling, cod were sacrificed with a blow to the head. Blood samples were extracted into 1 ml syringes containing heparin to prevent clotting. The blood samples were immediately transferred into eppendorf tubes held on ice before centrifuged for 5 min at 1000 x g to separate the plasma from the blood cells. The plasma was transferred into cryotubes while the blood cells remained in the eppendorf tubes. Both plasma and blood cells were frozen on liquid nitrogen. The total length and weight of cod sampled in the inner (Table 2.2) and the outer (Table 2.3) Oslofjord was measured, as well as the weight of the intestine, gonad, liver and the remaining tissue after removing the viscera. The presence of external or internal visible parasites or other abnormalities were registered. Gender was determined from visual inspection of the gonads. To dissect, a scalpel was used to cut open the fish from the anus up to the pectoral fin. To prevent bile from contaminating the liver, a syringe was used to remove bile from the gall bladder before dissecting out the liver. Bile was transferred into 0.5 ml eppendorf tubes, while three pieces of each liver were sampled (from the same region of the liver for all individuals) and transferred to cryotubes. Muscle pieces from same region on each individual were sampled using a scalpel and transferred into cryotubes. All of the samples were stored in liquid nitrogen. The cranium was opened and the otoliths (two from each individual) were taken out and kept at 4°C. When back at the laboratory the samples were transferred from liquid nitrogen into a -80°C freezer and stored until analysed.

Table 2.2: Sampled cod in the inner Oslofjord; length in centimetres (cm), weight in grams (g), min: minimum, max: maximum.

	Length			
	Min	Mean	Median	Max
Female	37	46.2	46	58
Male	35	47.2	46.5	65
Total	35	46.7	46	65
	Weight			
Female	465	942.8	822	1790
Male	388.2	1121.8	870	2748.3
Total	388.2	1027.8	850.5	2748.3

Table 2.3: Sampled cod in the outer Oslofjord; length in centimetre (cm), weight in grams (g), min: minimum, max: maximum.

	Length			
	Min	Mean	Median	Max
Female	40	49.9	51	59
Male	43	49.6	48.6	59.5
Total	35	49.8	49.9	59.5
	Weight			
Female	481	1158.6	1193.3	1691.5
Male	731	1156.8	1014	1963.6
Total	388.2	1157.8	1138.9	1963.6

2.3 Age, condition factor, liver somatic index and gonad somatic index

Age was determined by otolith reading according to the method from Williams et al. (1974). To determine the age of the cod, opaque and hyaline zones in the otoliths were visually examined under a binocular microscope. Rings formed during periods of slow growth (late fall and winter) appear as brown, hyaline zones, while growth increments formed during periods of rapid growth (spring and summer) appear as white, opaque zones. The otoliths were broken at the nucleus and filed before slightly burned with an ethanol-lamp. Glycerol was added to enhance the contrast between the opaque and the hyaline zones. One of the two pieces was examined using a lens with flexible side light. For all individuals, the condition factor, K, was calculated using the formula: $K=weight/length^3 *100$, LSI was calculated using the formula: LSI=liver weight/somatic body weight *100 and GSI was calculated using the formula: GSI=gonad weight/somatic body weight *100 (Schlenk et al., 2008).

2.4 Sample treatment

2.4.1 Bile preparation

The preparation of bile prior to HPLC analyses of PAHs metabolites was conducted according to the method in Krahn et al. (1992) and in further detail described in Grung et al. (2009). The laboratory work was performed at the Norwegian Institue for Water Research (NIVA). Bile samples were thawed and kept on ice. A volume of 20 µl bile and 10 µl triphenylamine

(internal standard) were added to eppendorf tubes and carefully mixed. Each of the tubes was weighted before adding the enzyme β-glucuronidase/arylsulfatase and 50 µl distilled water. Control samples with different concentration (1:100 and 1:1000) were included as reference samples. Further, the samples were incubated in a heating cabinet for 60 min at 37°C. After incubation, 200 µl methanol was added before centrifuging the tubes for 10 min at 13 000 x g in room temperature. The supernatant was transferred to HPLC-tubes and stored at –20°C prior to HPLC-analysis (see section 2.5.1).

2.4.2 Liver preparation

The purpose of the liver preparation, was to separate the liver tissue into a cytosolic and a microsomal fraction. The cytosolic fraction was used for measuring MT concentration (section 2.6.3), while the microsomal fraction for EROD activity (section 2.6.4). The preparation was conducted according to the method in Dignam (1990). To standardise the samples, liver pieces of approximately 1 g were weighed and transferred into homogenisation tubes containing 4.5 ml ice-cold homogenisation buffer (0.1 M potassium phosphate buffer, 0.15 M KCl, 1 mM DTT and 5% glycerol, with pH 7.8). The samples were homogenised using a Potter-Elvehjem homogeniser with a rotation speed of 6000 rpm and the teflon pistil was vertically moved up and down ten times before the homogenate was centrifuged for 30 min at 10 000 x g (4°C). Further, the supernatant was centrifuged for 120 min at 50 000 x g (4°C) and aliquoted into 1.5 ml eppendorf tubes before stored at -80°C. The remaining pellet (the microsomal fraction) was resuspended in 1.5 ml microsomal buffer (homogenisation buffer with 20% glycerol) and aliquoted into 0.5 ml eppendorf tubes before stored at -80°C.

2.4.3 Blood preparation

To be able to measure the ALA-D activity (section 2.6.2), the blood cells were homogenised. The red blood cells were thawed on ice before two-fold diluted in dilution buffer (0.05 M phosphate buffer with pH 7.0 and 0.5 ml triton X-100) and manually homogenised using a glass pistil before centrifuged for 15 min at 10 000 x g (4°C). For each sample, the pistil was thoroughly cleaned to avoid contamination. The supernatant from the homogenate was diluted three-fold time with dilution buffer and 50 µl were aliquoted into eppendorf tubes. The samples were immediately analysed for ALA-D after preparation.

2.4.4 Muscle preparation

The preparation was based upon homogenisation and centrifugation of the muscle tissue, since acetylcholinesterase (AChE) is a membrane-bound enzyme. The preparation and the following measurement of AChE activity (section 2.6.5) was conducted according to the method in Ellman et al. (1961) modified by Herbert et al. (1995). Approximately 1 g of muscle was weighed out and immediately transferred to 7 ml tubes with ice-cold tris-buffer (potassium phosphate buffer, 0.1 M, pH 7.2) to a total volume of 5 ml. The samples were homogenised using a PRO 200 Ultra-Turrax-Homogenisator with a generator of 7 mm x 75 mm and a speed of 25 000-35 000 rpm for 90 sec. After the homogenisation, the tissue was centrifuged for 15 min at 5 000 x g (4°C) before the supernatant was aliquoted into 1.5 ml eppendorf-tubes and stored at -80°C.

2.4.5 Isolation of total RNA from liver tissue

To be able to measure relative gene expression by RT-qPCR (section 2.7), total RNA was isolated from the liver samples using the MoleStrips RNA Tissue Kit and the GeneMole instrument (Mole Genetics) according to the manufacturer's instructions. Due to problems with the isolation process (difficult to obtain sufficient concentration in the isolate) including time and cost limitations, 49 individuals were analysed (Table 2.4).

Table 2.4: Sampled cod analysed for gene expression; no: number of cod individuals.

Area	Females (No)	Males (No)
Inner Oslofjord	13	13
Outer Oslofjord	12	11
Total	25	24

To standardise, samples were randomised and approximately 10 mg of tissue were weighed out per cod liver. The sampling was performed on dry ice and RNase AWAY® (Invitrogen) was used to wash equipment, gloves and benches during the isolation process to prevent RNA degradation. After sampling, the tissue was immediately transferred to 2 ml homogenisation tubes containing Mole Beads and 500 µl 80% ethanol. To achieve a successful RNA-isolation, the ethanol treatment is essential because of the high fat content in cod liver. The samples were homogenised using the Precellys 24 (Bertin Technologies) for 2x20 sec at 6000 rpm before being centrifuged for 1 min at 10 000 rpm. The supernatant was aspirated and the

tubes were placed in a fume hood for 5 min to evaporate the ethanol. After the evaporation step, 385 µl lysis buffer (modified from the protocol, 10 µl extra was added because of bubbles in the tube) was added and the samples were further homogenised in precellys 24 for 2 x 15 sec at 6000 rpm before centrifuged for 30 sec at 10 000 rpm. 350 µl supernatant was transferred to MoleStrips™ sample tubes and 5 µl 4% of glycogen solution was added before running the program "RNA isolation tissue with DNase" in the GeneMole® machine with MoleStrips™ RNA tissue reagents. The purity and integrity of RNA are important factors for the overall success of mRNA analyses (Fleige et al., 2006a). The concentration and purity of RNA was measured in 2 µl isolate by absorbance using a SynergyMx plate reader (BioTek) and the software Take3. Contamination from proteins can be estimated by the 260 nm (RNA) to 280 nm (protein) ratio. Similarly, contamination from organic compounds can be estimated by the 230 nm to 260 nm ratio. Samples with a ratio > 1.8 are considered acceptable, and only these samples were further analysed. The integrity of the isolated RNA was examined by inspection of the 18S and 28S ribosomal subunit by gel electrophoresis. This was performed on a Bioanalyzer480 machine with the RNA 6000 LabChip technology and the RNA 6000 Nano Kit. The procedure was performed according to the manufacturer's instructions. For more detailed description of the integrity measurements see appendix. To evaluate the results from the Bioanalyzer, the electropherograms were examined both visually and based on the RIN (RNA integrity value) value. To ensure that the isolate were not fragmented, only samples with distinct 18S and 28S peaks were used for RT-qPCR analysis.

2.5 Chemical analyses

2.5.1 PAH metabolites

For preparation of bile before analysis, see section 2.4.1. The HPLC-analysis was conducted by Merete Grung at NIVA. The HPLC system used, consists of a Waters-separation module 2695 with a 2475 fluorescence detector and a PAH C18 (4.6 x 250 mm) column with 5 µm particles (Waters). The analysis starts with acetonitrile and water in a gradient from 40:60 to 100% acetonitile in 30 min. The flow-through speed was 1 ml/min and the column held 35°C. The fluorescence peak was measured for the components; OH–phenanthrene, OH–pyrene and 3-OH-B(a)P.

2.6 Biochemical analyses

2.6.1 Protein concentration

The protein measurement assay was conducted after the method in Lowry et al. (1951). To standardise the biochemical assays, the total protein concentration needs to be measured. Protein levels were measured in the cytosolic fraction (for MT), microsomal fraction (for EROD), muscle homogenate (for AChE) and in blood homogenate (for ALA–D). The samples were thawed on ice and diluted in ice-cold 0.1 M Tris buffer (13.2 g Tris HCl and 1.94 g Tris base with a pH 8.0 at 4°C) to a concentration within the linear part of the standard curve. The standard stock solution (1.5 mg/ml) was made from bovine serum albumin in Tris buffer. To make the standard series, the stock solution was diluted in Tris buffer to concentrations of 1.0, 0.5, 0.25 and 0.125 mg/ml. A volume of 10 µl of Tris buffer (blank), standard or sample were added to a flat bottom microtiter 96-wells plate in triplicates (except from four blank replicates). The plates were held on ice until adding 25 µl of reagent A (alkaline copper solution) and 200 µl of reagent B (diluted Folin reagent) into each well (both reagents from BioRad). The plates were incubated for 15 min at room temperature before reading the absorbance at 750 nm was measured using the plate reader SynergyMx (BioTek) with the software Gen5. Protein concentrations in the different homogenates were calculated from the linear part of the standard curve.

2.6.2 EROD activity

The method is based on the 7-ethoksyresorufin *O*-deetylase (EROD) method described by Burke et al. (1974) and modified to use for 96-well plates, based on Eggens et al. (1992). Measurements of EROD activity were performed by analysing the microsomal fraction of the liver cells (see section 2.4.3). The samples were randomised and thawed on ice. To be able to adjust for any differences between the plates, the concentration of both the standard (1 µM Resorufin) and 7-ethoxsyresorufin (0.2 µM) was calibrated by absorbance reading at 572 nm and 450 nm, respectively. All of the solutions were stored in the dark. Prior to the analysis, the protein concentrations (see section 2.6.1) in the samples were measured to standardise the measurements of the enzyme activity in approximately equal amounts of protein (1.5 to 2.0 mg/ml). This was achieved by diluting the samples 12-fold in ice-cold potassium phosphate buffer. The working solution was made from 50 ml potassium phosphate buffer (0.1 M, pH 8.0) with 3 µM ethoxyresorufin. A standard series was made from resorufin with

concentrations of 0.64, 0.32, 0.16, 0.8, 0.4 and 0.2 µM. Each standard concentration was measured in duplicates, as well as eight replicates of blanks (potassium phosphate buffer) and six replicates (50 µl) of each sample where plated out (see appendix for plate layout). Three of these replicates where spiked with 10 µl resorufin (0.32 µM) to avoid quenching. 200 µl working solution and 25 µl NADPH (2.4 µM) were pipetteted to all wells, except from the wells containing resorufin. NADPH was added immediately after thawing. The reaction were read in eight steps (total of 4 min) using a fluorescence plate reader (excitation at 530 nm and emission at 590 nm). All of the steps were carried out in the dark.

2.6.3 MT concentration

This analysis was conducted according to the method in Olafson et al. (1991). The lab work was performed at NIVA. The cytosolic fraction (see section 2.4.3) was analysed for MT content in liver. The cytosol was thawed on ice before diluted ten-fold with ice-cold dilution buffer (0.9% NaCl) before denatured by using a digital heat-block (VWR) for 4 min at 95°C. Proteins will generally denaturate at such high temperatures, but MT is considered to be very tolerable for heat and will theoretically be the only remaining protein in the cytosol. After the denaturation step, the samples were held on ice before centrifuged for 15 min at 10 000 x g (4°C). The supernatant was transferred into new tubes and mixed well before analysed in the Polarograph. The denatured cytosol was transferred to PARC glass with 300 µl Triton X-100 and 10 ml electrolyte (0.322 g/l Co(NH3)6Cl3, 53.4 g/l ammonium chloride and 154 ml/l 25% NH4OH). The electrolyte solution was stored in a dark bottle at 4°C and tempered to room temperature prior to use. Rabbit MT (Sigma-Aldrich) was used as a standard with 50 µg/ml 0.9 % NaCl, and a standard curve was measured at the start of each analysis day by adding 10, 20, 30 and 40 µl of Rabbit MT. Depending of the concentration in the sample to be able to obtain a measurement within the standard curve a volume between 150-400 µl of sample was added to the solution. Nitrogen gas was bubbled into the solution for 1 min prior to the measurement to reduce the amount of dissolved oxygen which can potentially influence the result. The software used for this analysis was 757 VA Computrace v1.0.

2.6.4 ALA-D activity

For blood preparation, see section 2.4.2. The analysis was conducted according to the method in Hodson et al. (1977) and modified by Hylland (2004). The samples were randomised prior

to the analysis. Both the dilution buffer and the precipitation solution were made in advance and stored at 4°C. The standard solution was stored at -80°C. Both ALA- and Ehrlich-reagents were freshly made each day, and the DMAB was added to the Ehrlich-reagent before use. Each sample was measured in six replicates; three were added 200 µl dilution buffer while the other three were added 200 µl ALA-reagent. The samples were then mixed well and incubated for precisely 2 hrs at 25°C. After two hours, precipitation reagent (4.0 g trichloroacetic acid and 2.7 g HgCl2 in dH2O to 100 ml) was added to all of the samples except the standard to stop the reaction. To standardise the reaction time, the adding of reagents were carefully calculated. After the precipitation solution was added, the samples were incubated for 5 min before centrifuged for 5 min at 10000 x g in room temperature. The standard was made of PBG from a stock solution of 40 µg PBG/ml dilution buffer and a standard series with concentrations of 0, 50, 100, 200, 400 and 800 ng PBG/µl. 150 µl of each standard was added in duplicates into a flat bottom microtiter 96-wells plate. For each sample, the three replicates with ALA and the three with dilution buffer were plated out. A volume of 150 µl modified Ehrlich`s reagent was added to all of the wells and mixed by carefully agitating the plate by hand before incubating the plate for 15 min at room temperature. After incubation, the absorbance was read at 550 nm in plate reader. See appendix for more details.

2.6.5 Acetylcholinesterase (AChE) activity

Muscle homogenate (section 2.4.4) was randomised prior to the analysis. After thawing, the homogenate was ten-fold diluted in Tris-buffer and mixed well. A volume of 50 µl was plated out in four replicates for each sample and 250 µl of AChE working solution (Tris-buffer, 0.075 M ATC, 0.01 M DTNB) were added into all the wells in a flat bottom Microtiter 96-wells plate. Tris buffer (blank) was plated out into four wells. The plates were incubated for 5 min at 25°C prior to kinetics measurements by reading the absorbance at 414 nm every 2 min for a total of 10 min. To avoid sedimentation during the kinetic measurements, the plates were stirred carefully prior to each reading. Fresh reagent mixtures were made each day and stored in the dark. See appendix for more details.

2.7 Gene expression

The real time quantitative polymerase chain reaction (RT-qPCR) assay is a quantitative PCR method that determines the original amount of mRNA molecules for a given target gene. This

is achieved by measuring the fluorescent signal and the increase of this signal produced per amplification cycle (Sugden et al., 2008). In addition to the isolation of RNA (section 2.4.5), this method is based upon the following steps; reverse transcription from RNA into complementary DNA (cDNA), amplification of cDNA using PCR and real time quantification of the amplification products (Nolan et al., 2006).

2.7.1 Complementary DNA (cDNA) synthesis

In order to measure the relative rate of gene expression, a standard series was made from a stock solution of 400 ng RNA. This stock was prepared from a mix of all the RNA isolates to ensure that expression levels from all genes were represented. The RNA concentration in the stock was two-fold higher than the concentration input for each of the samples for complimentary (cDNA) synthesis. To be able to compare the relative expression levels of the different genes between the samples, 20 ng/µl of RNA was the standardised input for the cDNA synthesis. All of the samples were diluted in RNase-free water to gain this concentration. The standard curve was made by diluting the highest standard in RNase-free water to concentrations of 200, 100, 50 and 25 ng RNA. To quality check these concentrations, the standard stock solution and the samples were measured on a plate reader using Take3. For cDNA synthesis, the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems) without RNase inhibitor was used. A total volume of 20 µl containing 200 ng RNA template, 1X RT buffer, 4mM dNTPs, 50 µM of RT random primers, 2.5 µM oligo-dT-primers and 50 units of MultiScribe reverse transcriptase, were used for the cDNA synthesis. The reaction was performed in PCR 96-wells clear plates (Axygen). In addition to standard and samples, two controls were included; no enzyme control (NEC) and no template control (NTC) (see appendix for plate layout). This was to control for contamination of foreign transcriptase or RNA molecules. After adding all of the components to the plate, the plate was sealed with a lid and centrifuged to spin any content down and to eliminate air bubbles. The plates were held on ice until ready to incubation. The reaction mixture was incubated in a thermal cycler (Eppendorf) for 10 min at 25°C, 120 min at 37°C and 5 min at 85°C. After the incubation, the plates were kept on ice until stored at -20°C prior to RT-qPCR.

2.7.2 Real time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR primers for selected genes were chosen based on a literature search (Lie et al., 2009a; Lie et al., 2009b; Olsvik et al., 2008; Søfteland et al., 2010; Olsvik et al., 2010; Olsvik et al., 2009; Lie et al., 2009c) and ordered from Invitrogen (Table 2.5).

Table 1.5: Genes examined by RT-qPCR with gene name, Abb: gene symbol, nucleotide sequences for the forward and reverse primer, primer melting temperature (TM), Acc.No: GenBank accession number and size of amplicon product (bp: base pair).

Gene	Abb	Forward (5'-3')	Reverse. (5'-3')	TM (°C)*	Acc. No.	Size (bp)
β-actin	ACTB	CACAGCCGAGCGTGAGATT	ACGAGCTAGAAGCGGTTTGC	55.2	EX739174	95
Elongation factor 1A	EF1A	CCCTGTGGAAGTGGCTGAAG	CATCCAAGGGTCCGTATCTCTT	55.6	EX721840	93
Cytochrome P450 1A	CYPa	CCTTGACCTCTCGGAGAAAGAC	CGCCCCGCTAGCTATAGACA	56.7	EX725014	145
Heat-shock protein 70	HSP70	CCCCTGTCCCTGGGTATTG	CACCAGGCTGGTTGTCTGAGT	58.7	BG933934	121
Metallothionein	MT	CCTTGCGACTGCACCAAGA	CAGTTTAGGCAGGTGCATGATG	54.2	EX721491	68
Glutathione S-transferase	GST	GTCCCCCTGCTGCCATTC	CCTCCATACACCGCCACCTA	58.6	EX730032	126
Heme- oxygenase 1	HO-1	AGAGAACACAGGGCTGATGTTGA	CGGGTGGCTGCTGTTATTGT	56.1	EX738947	133
Vitellogenin A	VtgA	AGACTGGCCTGGTCGTCAAA	GCGAGGATAGAGGCAGGGAT	57.9	AF284035	121
Uridinediphosphate -glycurono -syltransferase	UGT	GTAAAAATAATGAAGTGGCTACCTCAAA	GAACGCCGTGGCAGATG	50.8	EX722276	115

*: 5°C below TM for the forward or reverse primer with the lowest MT.

Primer stock solution (100 µM) was stored at - 20°C prior to the analysis. Of the nine selected genes, two reference genes were included; Elongation factor 1α and β-actin for control. SYBR Green I master mix (Roche) together with the LightCycler® 480 (Roche) instrument and LightCycler®480 96-multiwell plate (Roche) were used for the RT-qPCR and performed according to the manufacturer's instructions (protocol A for 96 wells plate). SYBR Green is a specific dye for double-stranded DNA, and during each phase of DNA synthesis, the SYBR Green binds to the amplified PCR product to detect amplification by fluorescence. The DNA polymerase is inactive at room temperature because of the heat-labile blocking groups on some of the amino acid residues, and therefore there is no elongation during the period when primers may non-specifically anneal. A pre-incubation step at 95°C was performed to denaturate the blocking groups and to activate the polymerase. The PCR reaction mix for one

reaction was made from 10 µl of ready-to-use master mix and 6 µl PCR-graded water mixed in an RNase-free eppendorf tube. This ready-to-use master mix contains FastStart Taq DNA polymerase, reaction buffer, dNTP mix, SYBR green and $MgCl_2$. Into this, 1 µl forward and 1 µl reverse primer (0.5 µM) for the selected gene were added For each gene, 18 µl PCR mix was added to all wells as well as 2 µl of cDNA (the plate with cDNA product were kept at -20°C and taken out for each gene). To prevent evaporation and contamination, the plates were sealed with sealing foil. The plates were centrifuged for 2 min at 1500 x g before kept on ice prior to the PCR in the Lightcycler. The plate setup was identical with the one for cDNA transcription. For Lightcycler settings, see appendix. Fluorescence curves that cross the crossing point (Cp) after the 15th and before the 35th cycle were considered to be successful.

2.8 Statistics

All assays, except from the RT-qPCR data, were performed using the program Jmp 9.0.0 and the figures were made in Graphpad prism. To determine if we should use parametric or none-parametric tests, the data were analysed for normal distribution (Siegel, 1957) by examine q–q plot for the four groups (inner Oslofjord females, inner Oslofjord males, outer Oslofjord females, outer Oslofjord males) and homogeneous variance by Levene's test (Levene, 1960). The data which met these requirements (condition factor, LSI, 3-OH-B(a)P, MT and EROD), were analysed by a two-way ANOVA. GSI, OH-phenathrene, OH-pyrene, ALA-D, and AChE were analysed by Mann-Whitney tests for significant differences between females and males within inner Oslofjord, for females and males within outer Oslofjord, between females from the two areas and between males from the two areas. For age differences between inner and outer Oslofjord, the data were analysed with by a Wilcoxon test.

For the RT-qPCR, after calculating the Cp (crossing point) value, the relative expression was analysed by using the Relative Expression Software Tool (REST) program. The program is based on a randomisation test where the Cp values are repeatedly (in this case 2000x) and randomly reallocated between the two groups. This test is parametric but there is no requirement for normal distribution or homogeneous variance due to the randomisation (Pfaffl et al., 2002). This assay compare gene expression for two groups against each other arranged as treated group and untreated group (treated = inner Oslofjord, untreated = outer Oslofjord). To calculate the relative gene expression for each sample, the samples were standardised against the reference gene. The expression of a reference gene is thought to be

unaffected by toxicants. For all these testes above, the significance level was set to be 0.05, and values below this were accepted as statistically significant.

To analyse if there were correlation between the measured variables, Sperman`s rank correlation tests were carried out for each group. This is a none-parametric test that examines the relationship between variables by ranking them (Zar, 1999). Not to make false correlations, all outliers were removed prior to this analysis. Only the variable with significant correlation were brought in the results, with exception from the biomarkers measured at both protein and gene expression level. There was also performed a bonferroni-correction and this led to the p-value of 0.0026. Only p-values below this was accepted as statistical significant for the Sperman`s rank correlation tests. All the correlations with p-values <0.05 were included in the appendix.

3 Results

3.1 Age, condition factor, liver somatic index and gonad somatic index

The age distribution of the cod sampled from inner and outer Oslofjord is presented in table 3.1. There were no statistically significant differences in age between areas (Wilcoxon; p = 0.11). Figure 3.1 indicates that cod had a smaller length and weight in the inner Oslofjord than in the outer Oslofjord. There was also seen from R^2 that 71% of the age explains the length (Fig 3.1A) while 68% of the age explains the weight (Fig 3.1B).

Table 3.1: Age determination from otholith reading with numbers of individuals for each age group measured in years (yrs). Inner: inner Oslofjord, Outer: Outer Oslofjord.

	Age groups (yrs)					
Area	1	2	3	4	5	6
Inner	1	12	21	6	-	-
Outer	3	9	12	9	5	2

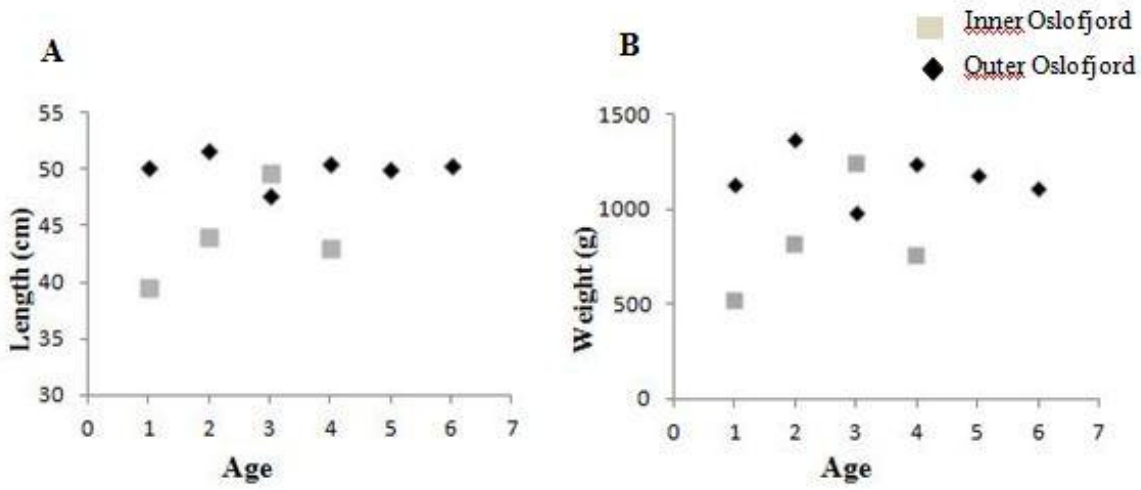


Figure 3.1A: Mean length (cm) (A) and mean weight (g) (B) as response variables and age measured in years as explanatory variable. Separated into inner and outer Oslofjord.

The individuals were larger in both length and in weight in the outer Oslofjord in comparison to the inner Oslofjord (Fig. 3.1) for all ages except from the three years old. There were too few individuals to test the one year olds, but the two year olds was significantly larger (length) in the outer than in the inner Oslofjord (Mann-Whitney; 0.0228). There were no

differences between the size (length) of the three year olds (Mann-Whitney; 0.5363) but a significant difference for the four year olds (Mann-Whitney; 0.036) where the cod was larger in the outer fjord.

For the condition factor there were no significant differences between inner and outer Oslofjord or between sexes (two-way ANOVA; table 3.2). There was larger variability in the data for cod from the outer Oslofjord than cod from the inner Oslofjord for both sexes (Fig. 3.2A).

Table 3.2: Two way analysis of variance (ANOVA) for condition factor. Factor: variable, DF: degrees of freedom, F-ratio: Fisher ratio.

Factor	DF	F-ratio	P-value
Sex	1	1.4694	0.2
Area	1	0.2763	0.6

There were no significant differences in LSI between cod from the inner and outer Oslofjord, nor between sexes (two-way ANOVA; table 3.3). There was a somewhat larger variability of data for females in the outer than in the inner Oslofjord (Fig. 3.2B).

Table 3.3: Two-way ANOVA for liver somatic index. Factor: variable, DF: degrees of freedom, F-ratio: Fisher ratio.

Factor	DF	F-ratio	P-value
Sex	1	0.4288	0.5
Area	1	0.059	0.8

There was a significant difference in GSI between females from the inner and outer Oslofjord (Mann-whitney; P = 0.01) with higher GSI levels for females from the inner Oslofjord as seen in figure 3.2C. This was not the case for males, with no significant different for cod from the two areas (Mann–Whitney; p = 0.9). There was no significant difference between the two sexes in the inner (Mann-Whitney; p = 0.2) or in the outer Oslofjord (Mann Whitney; p = 0.7).

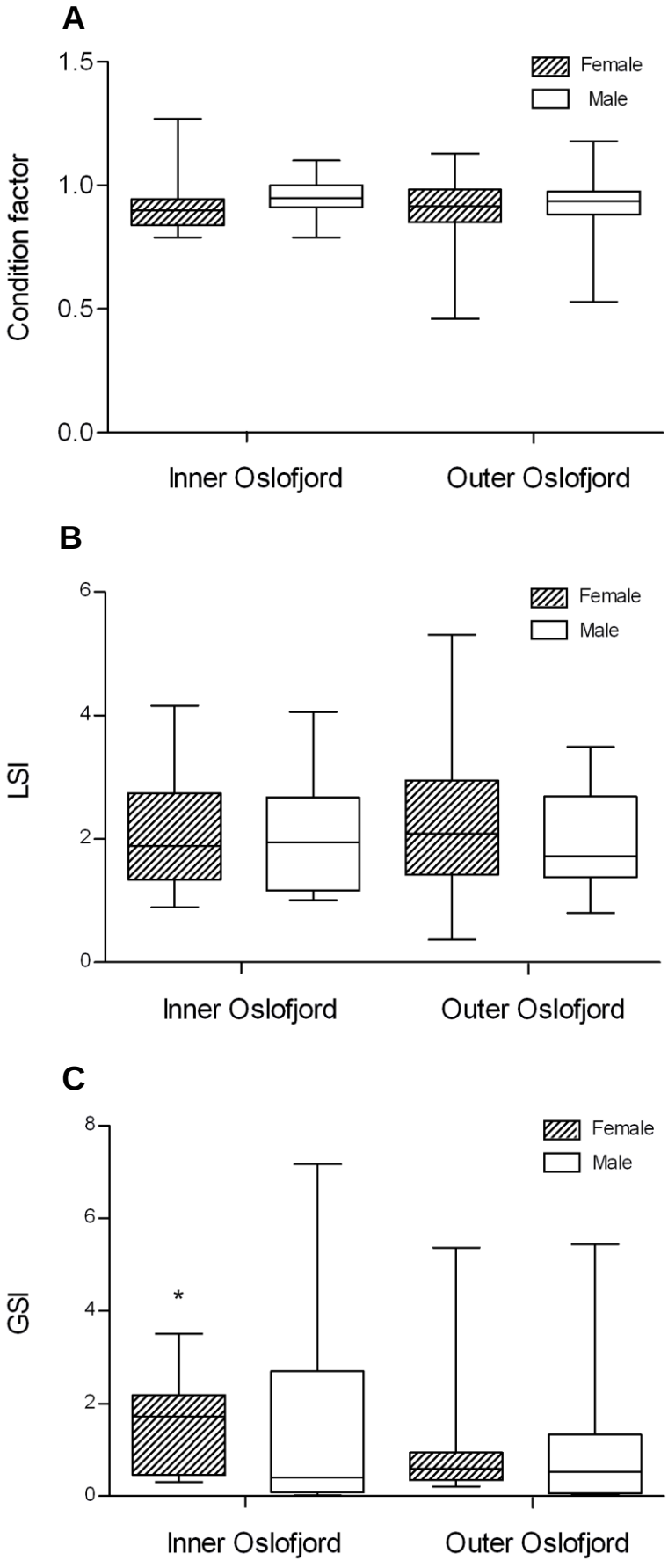

A
1.5
1.0
0.5
0.0
Condition factor
Female
Male
Inner Oslofjord
Outer Oslofjord
B
6
4
2
0
LSI
Female
Male
Inner Oslofjord
Outer Oslofjord
C
8
6
4
2
0
GSI
*
Female
Male
Inner Oslofjord
Outer Oslofjord

Figure 3.2: Condition factor (A), liver somatic index (B) and gonad somatic index (C) for cod from the two areas; median, quartiles, 10/90 percentile. The symbol * indicates significantly higher values compared with the other groups with a p-value < 0.05.

To detect which age group that contributed to this difference seen in GSI, analyses were preformed on cod individuals from the age groups two- and three-year olds for both females and males from inner and outer Oslofjord. The other age groups had too few individuals to run a statistical test. There were no statistical difference in GSI between two-year old females from inner and outer Oslofjord (Mann-Whitney; $p = 0.9$), but a significant difference between the three year old females from the two areas (Mann-Whitney; $p = 0.02$) with a higher value for inner Oslofjord females (Fig. 3.3A). There were no significant differences in LSI for the three year old females (Mann-Whitney; $p = 0.7$) although there was a higher spread of data for three year old males from the outer Oslofjord compared to the inner Oslofjord (Fig. 3.3B).

The same approach was followed for the males as for the females. There were no significant difference in GSI for neither the two-year olds (T-test; $p = 0.7$) or three-year old males (Mann-Whitney; $p = 0.4$) from the two areas. Although as seen from figure 3.3C, the spread of data was much higher for the three year olds males from the inner Oslofjord in comparison to the outer Oslofjord. The difference in LSI was near to statistically significant for three-year old males (Mann-Whitney; = 0.05) from the two areas with higher values for males from the inner Oslofjord as reflected in figure 3.3D.

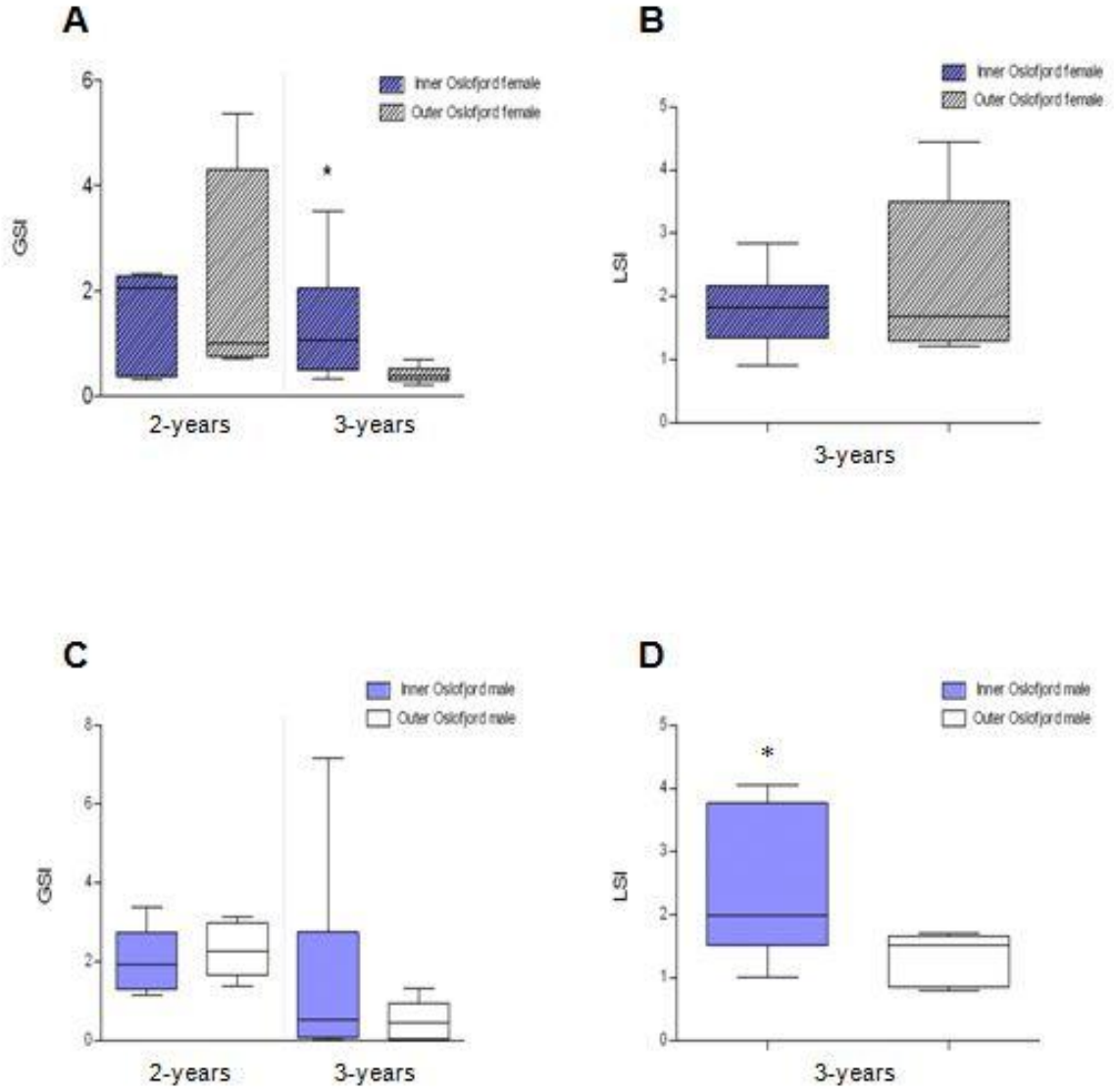


Figure 3.3: GSI measurements for two different age-groups (two and three year olds) of females and males (A and C, respectively) and condition factor calculated for the three year old female and male (B and D, respectively); median, quartiles, 10/90 percentile. The symbol * indicates significantly higher values with a p-value of 0.05 or below.

3.2 Chemical analyses

3.1.1 PAH metabolites

The detection limit for PAH metabolites was set to 2 ng/g bile. Samples below this level were excluded from the statistical analyses. There were found no significantly differences in OH-phenanthrene content in bile between females (Mann-Whitney; p = 0.9) or for males (Mann-

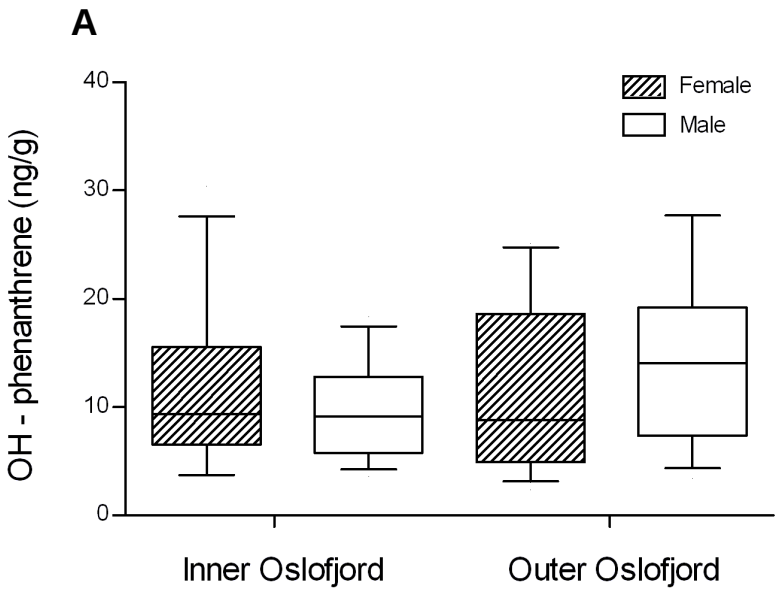
Whitney; p = 0.3) between the two areas. There were found no different concentration for sex within the inner Oslofjord (Mann-Whitney; p = 0.9) or for sex within the outer Oslofjord (Mann-Whitney; p = 0.2). These results are also reflected in figure 3.4A. For cod from the inner Oslofjord 90% of the females and 95% of the males had levels above detection limit, while among cod the outer Oslofjord 88% of the females and 94% of the males had levels above detection limit.

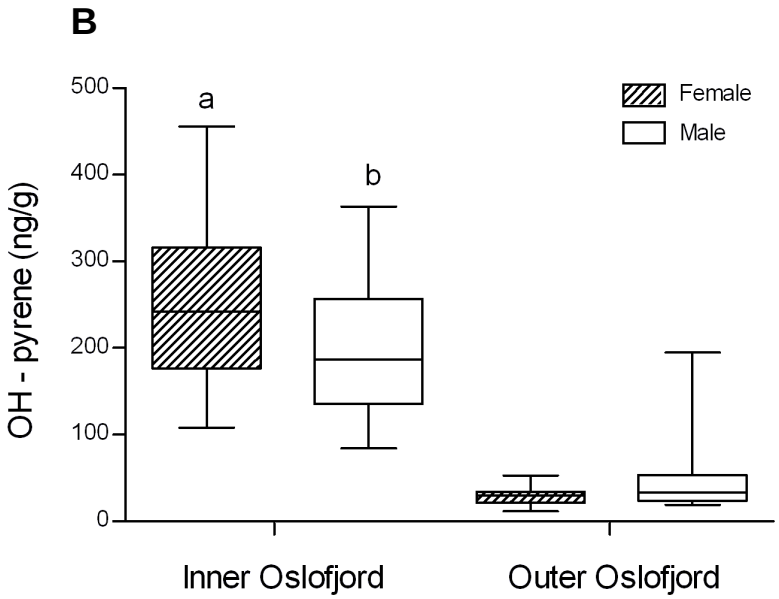
There were significantly different concentrations of OH – pyrene in bile of females (Mann-Whitney; p = 0.001) as well as for males between inner and outer Oslofjord (Mann-Whitney; p = 0.001). In the inner Oslofjord the concentrations were higher than in the outer Oslofjord (Fig. 3.4B). Within the inner Oslofjord there were significantly differences between concentration of OH-pyrene for female and male cod (Mann–Whitney; p = 0.045) and females had higher concentration than males as seen in figure 3.4B. In the outer Oslofjord there were found no significant difference for the two sexes (Mann-Whitney; p = 0.1).

There were no significantly different concentrations of the metabolite 3-OH-B(a)P between either area or sex (two-way ANOVA; table 3.4). As indicated from figure 3.4C, there was a higher spread of data for female cod from the outer Oslofjord. In the inner Oslofjord 42.8% of the females and 52.6% of the males had levels above detection limit, while in the outer Oslofjord 45.8% of the females and 43.8% of the males with OH-BaP content found above detection limit.

Table 3.4: Two-way ANOVA for hydroxyl-benzo(a)pyrene. Factor: variable, DF: degrees of freedom, F-ratio: Fisher ratio.

Factor	DF	F-ratio	P-value
Sex	1	0.2421	0.6
Area	1	2.2801	0.1


A
OH - phenanthrene (ng/g)
40
30
20
10
0
Female
Male
Inner Oslofjord
Outer Oslofjord


B
OH - pyrene (ng/g)
500
400
300
200
100
0
a
b
Female
Male
Inner Oslofjord
Outer Oslofjord

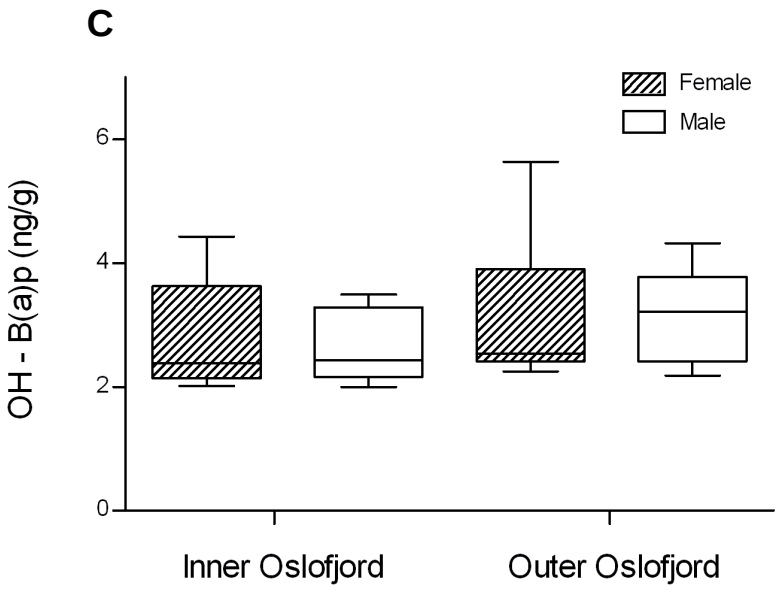

C
OH - B(a)p (ng/g)
6
4
2
0
Female
Male
Inner Oslofjord
Outer Oslofjord

Figure 3.4: Bile metabolites in male and female cod from the two areas. OH-phenanthrene (ng/g) (A), OH-pyrene (ng/g) (B), OH-B(a)P (ng/g) (C); Median, quartiles, 10/90 percentile. Groups with significant higher concentration of PAH in one area compared with the other is marked with "b", while group with both higher values compared with the other area and higher concentration than the other sex, is marked with "a".

3.3 Biomarkers

3.3.1 EROD activity

There were no significant differences in hepatic EROD activity for sex or area (two-way ANOVA; table 3.5). Although there were, as seen from figure 3.5, a higher spread of data for cod from the inner Oslofjord.

Table 3.5: Two-way ANOVA for EROD activity. Factor: variable, DF: degrees of freedom, F-ratio: Fisher ratio.

Factor	DF	F-ratio	P-value
Sex	1	0.0121	0.9
Area	1	0.8319	0.4

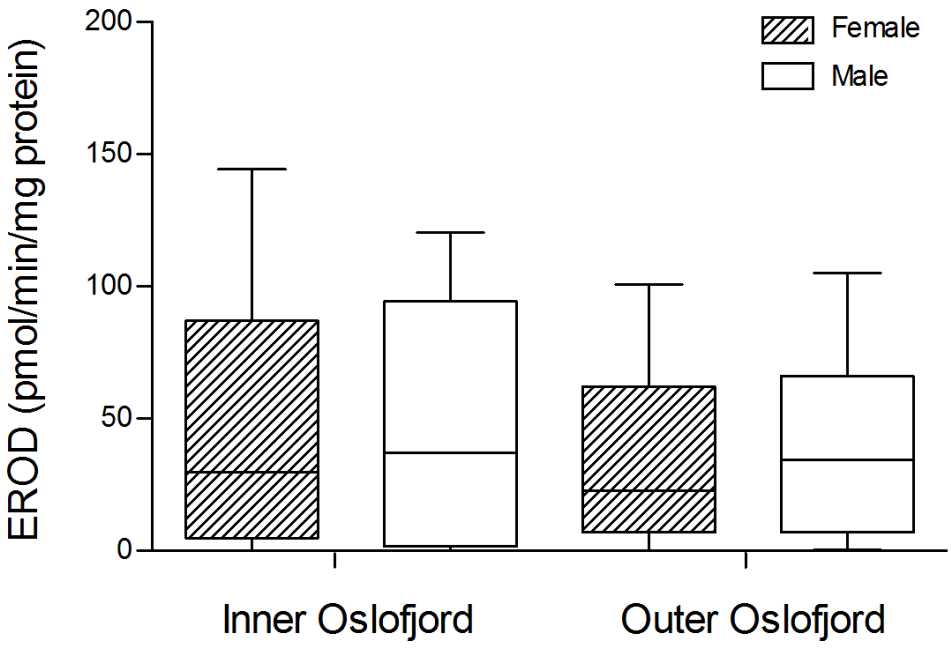


Figure 3.5: Hepatic EROD activity in female and male cod from the two areas; median, quartiles, 10/90 percentile.

3.3.2 MT concentration

There were no significant differences in hepatic MT concentration between the two areas or between females and males (two-way ANOVA; table 3.6). As seen from figure 3.6 and the low p-value of 0.06 between area, there seems to be a trend toward higher concentration of MT protein in liver of inner Oslofjord cod.

Table 3.6: Two-way ANOVA for hepatic MT activity. Factor: variable, DF: degrees of freedom, F-ratio: Fisher ratio.

Factor	DF	F-ratio	P-value
Sex	1	0.1362	0.7
Area	1	3.5399	0.06

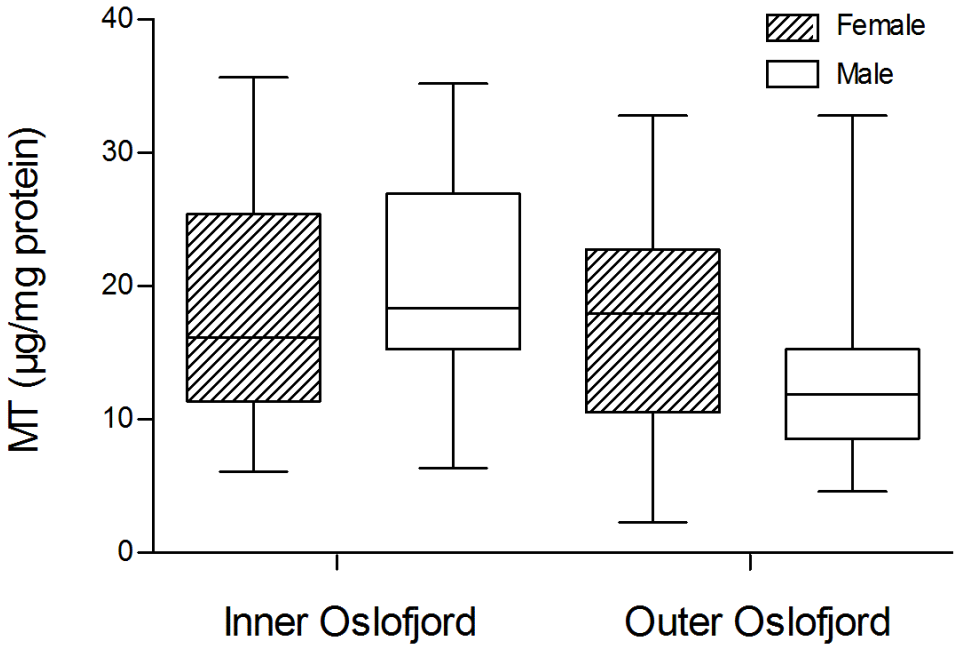


Figure 3.6: Hepatic MT concentration in female and male cod from the two areas; median, quartiles, 10/90 percentile.

3.3.3 ALA-D activity

There were no significant differences in the ALA-D activity between females from the inner and outer Oslofjord (Mann-Whitney; $p = 0.2$) or between males from the two areas (Mann-Whitney; $p = 0.3$). There were also no significant difference in ALA-D activity between females and males within the inner Oslofjord (Mann-Whitney; $p = 0.8$) or between females and males from the outer Oslofjord (Mann-Whitney; $p = 0.5$). There was a larger variability of data for males from both areas as seen in figure 3.7.

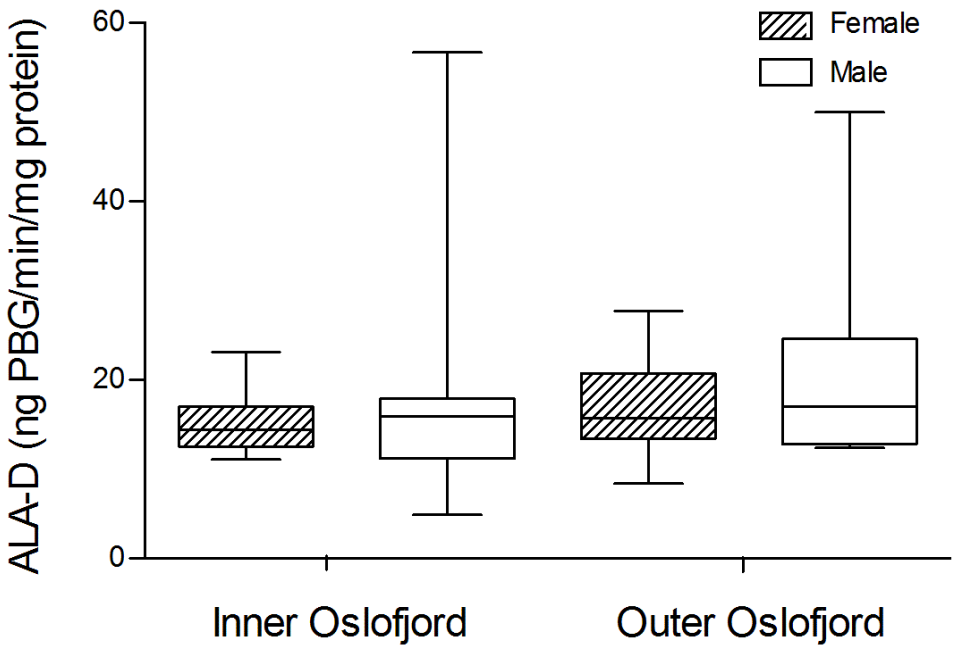


Figure 3.7: ALA-D activity in red blood cells in female and male cod from the two areas; median, quartiles, 10/90 percentile.

3.3.4 AChE activity

There were found significant differences in the enzyme activity between females from the inner and Outer Oslofjord (Mann-Whitney; p = 0.03) with a significantly lower activity in female cod from the outer Oslofjord as seen from figure 3.8. In male cod no such difference were found between the two areas (Mann-Whitney; p = 0.2). There were no significant differences in AChE activity between females and males from the inner Oslofjord (Mann-Whitney; p = 0.3) or between the two sexes from the outer Oslofjord (Mann-Whitney; p = 0.3).

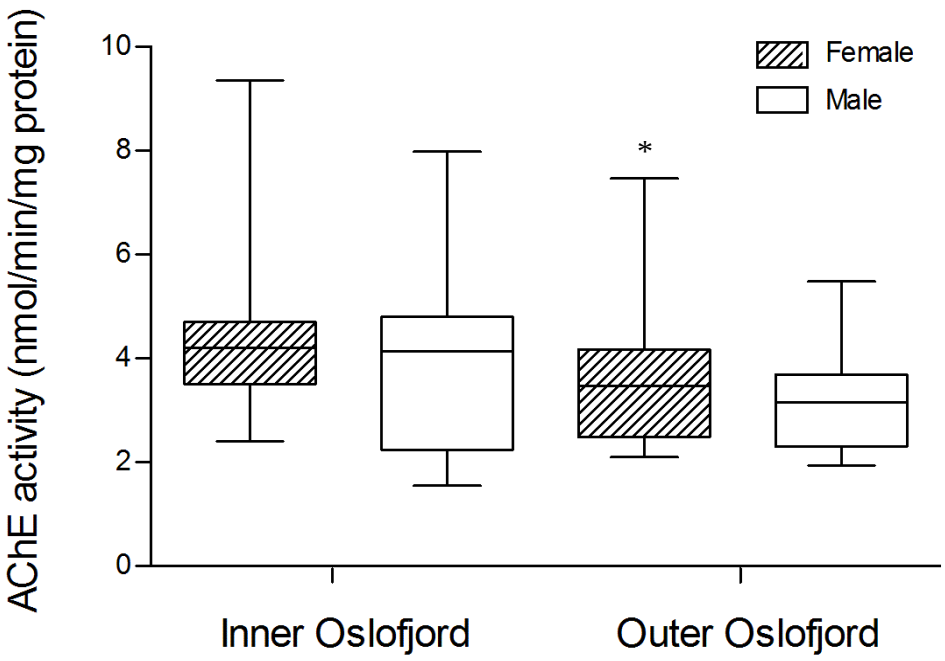


Figure 3.8: AChE activity in muscle of female and male cod from the two areas; median, quartiles, 10/90 percentile. The symbol * indicates significantly lower values than females in inner Oslofjord with a p-value < 0.05.

3.4 Gene expression

The RT-qPCR analyses of GST gene expression showed a melting curve with more than one peak. This gene was thereby excluded from the analysis since more than one product seemed to have been amplified (Nolan et al., 2006). EF1A and ACTB were originally used as reference genes. However, ten times higher concentration of primers was used for EF1A, hence only ACTB was used as a reference gene to standardise the gene expression levels of the other genes. The normalised results from the relative gene expression of selected genes measured by RT–qPCR, is presented in table 3.7 for differences between inner and outer Oslofjord. Differences between the expression for females and males within the inner and outer Oslofjord is presented in table 3.8 and 3.9 respectively**.** There were significant differences in gene expression of CYP1A for females from the inner and outer Oslofjord (REST; table 3.7) as well as for males between the two areas (REST; table 3.7). The gene expressions were higher for both sexes in the inner Oslofjord and thereby considered being

up-regulated (Fig. 3.9). None of the other genes had a significantly difference in gene expression between the areas. As seen in table 3.9 and figure 3.9 there could also seem to be a trend towards higher MT mRNA in the inner Oslofjord, although this was not significant. There was a higher spread of data for Vtg for males from the inner Oslofjord than in males from the outer Oslofjord as seen from the confidence interval (Table 3.7) and from the figure 3.9. Males in the inner Oslofjord and females in the outer Oslofjord also had a higher spread of data for HO-1 expression than the other groups (Fig. 3.9).

Table 3.7: Relative gene expression for males and females with inner Oslofjord as treated and outer Oslofjord as untreated area. Gene symbol: Cytochrome P450 1A (CYP1A), Metallothionein (MT), Vitellogenin (Vtg), Heme oxygenase-1 (HO-1), Uridine diphosphate glycuronosyltransferase (UGT) and Heat-shock protein 70 (HSP70). Sex: ♀: female, ♂: male, 95% C.I: 95% confidence interval, Exp.factor: expression factor, P: p-value, Result: UP: up-regulated.

Gene symbol	Sex	95 % C.I	Exp. factor	P	Result
Cyp1A	♀	0.508 – 11.603	2.29	0.003	UP
Cyp1A	♂	0.747 – 16.304	3.73	0.001	UP
MT	♀	0.261 – 4.029	1.23	0.4	
MT	♂	0.384 – 6.521	1.51	0.1	
Vtg	♀	0,001 - 19 871,192	1.83	0.7	
Vtg	♂	0.123 – 408.672	5.44	0.2	
HO – 1	♀	0.177 – 3.422	0.85	0.7	
HO – 1	♂	0.386 – 5.367	0.85	1.0	
UGT	♀	0.454 – 1.533	0.88	0.4	
UGT	♂	0.570 – 2.152	0.88	0.6	
HSP70	♀	0.769 – 1.832	0.37	0.4	
HSP70	♂	0.677 – 1.778	0.37	0.6	

There was a significantly difference in gene expression of CYP1A between females and males within the inner Oslofjord (REST; table 3.8) with a higher expression in males (Fig. 3.9). In the outer Oslofjord no significant difference in CYP1A expression were seen between the two sexes (REST; table 3.9). For Vtg there was significantly different expression for outer Oslofjord females and males with a significantly higher expression for females. There were no significantly differences in gene expression for sex in the outer Oslofjord (Table 3.8).

Table 3.8: Relative gene expression between females and males within inner Oslofjord. 95% C.I: 95% confidence interval, Exp.factor: expression factor, P: p-value, Result: UP: up-regulated. See table 2.5 for complete gene names.

Gene symbol	95 % C.I	Exp. factor	P	Result
CYP1A	0,346 - 1,922	0.605	0.011	UP
MT	0,105 - 2 637,177	0.823	0.5	
Vtg	0,002 - 47 474,032	15.662	0.07	
HO-1	0,101 - 2,082	0.701	0.7	
UGT	0,534 - 1,673	0.899	0.6	
HSP70	0,649 - 1,930	1.039	0.8	

Table 3.9: Relative gene expression between females and males within outer Oslofjord; 95% C.I: 95% confidence interval, Exp.factor: expression factor, P: p-value, Result: UP: up-regulated. See table 2.5 for complete gene names.

Gene symbol	95 % C.I	Exp. factor	P	Result
CYP1A	0.142 - 4.262	0.81	0.5	
MT	0.146 - 6.554	0.829	0.6	
Vtg	0,035 - 18 575,807	38.47	0.005	UP
HO-1	0.023 - 14.164	0.731	0.6	
UGT	0.239 - 4.247	0.981	0.9	
HSP70	0.267 - 2.605	0.811	0.3	

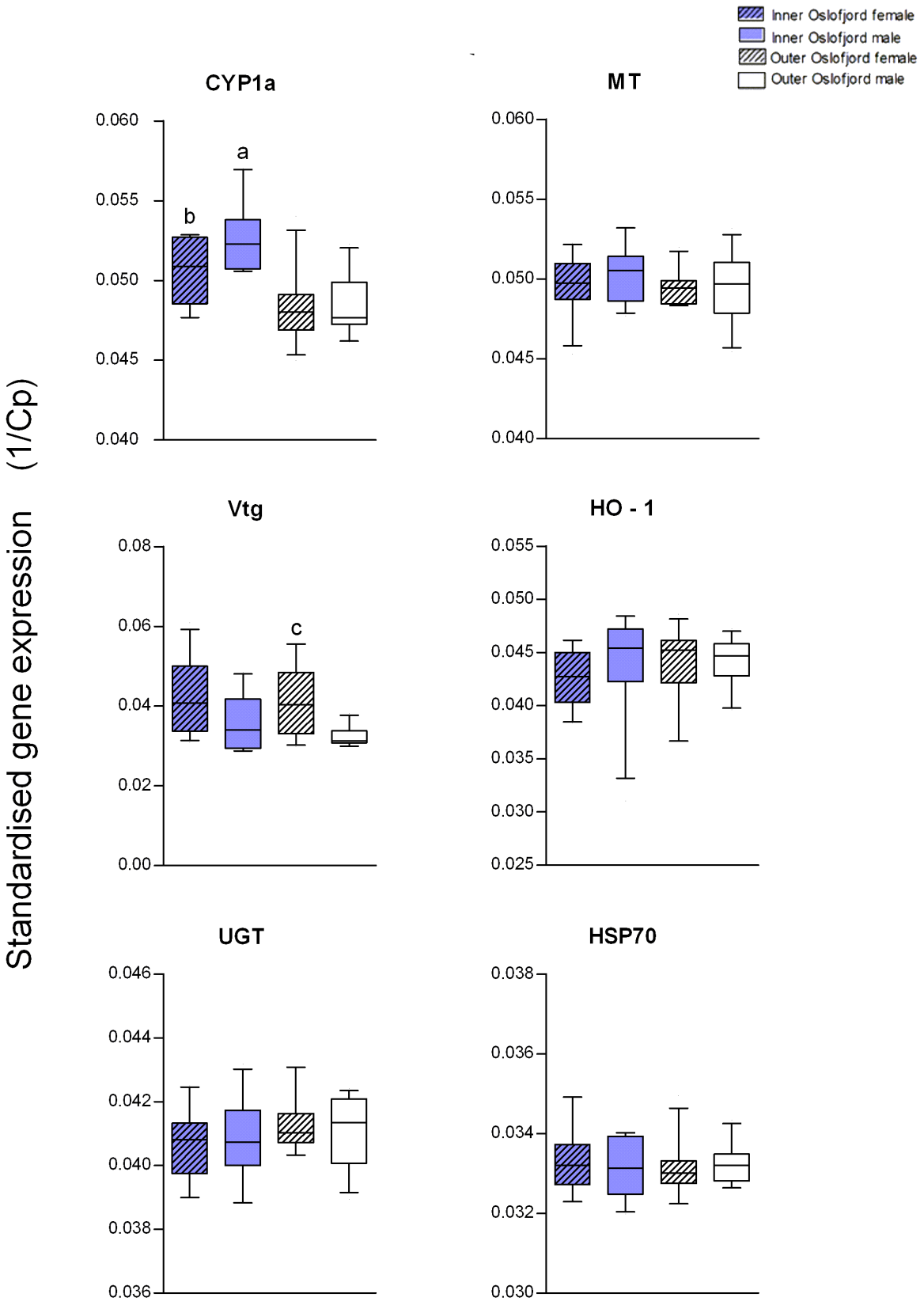

Inner Oslofjord female
Inner Oslofjord male
Outer Oslofjord female
Outer Oslofjord male
CYP1a
MT
Vtg
HO - 1
UGT
HSP70
Standardised gene expression (1/Cp)
a
b
c

Figure 3.9: Relative gene expression presented as inverted Cp (1/Cp). Cp value is defined as the point at which the fluorescence increases above background fluorescence in terms of number of cycles. Genes presented; cytochrome P450 1A (CYP1A), metallothionein (MT), vitellogenin (Vtg), Heme–oxygenase 1 (HO-1), Uridine diphosphate glucuronosyltransferase (UGT) and heat-shock protein70 (hsp70) for females and males from the inner and outer Oslofjord. Groups with significantly higher gene expression in one area compared with the other is marked with "b", while groups with both higher values compared with the other area and higher concentration than the other sex, is marked with "a". Groups with significantly higher levels than the other sex within one area is marked with "c".

3.5 Relationships between parameters

The results from the none-parametric Sperman`s rank order correlation are presented in table 3.10 for females and table 3.11 for males from the inner Oslofjord. The results from the outer Oslofjord are represented in table 3.12 for females and 3.13 for males. Generally, there were more variables that correlated in the inner than in the outer Oslofjord. Males from the inner fjord had most variables that correlated. None of the same variables correlated for the four groups, except for length and weight.

Table 3.10: Spearman's rank correlation for females from the inner Oslofjord; variables, Rs: Spearman`s rank correlation coefficient and p-value.

Variable	Variable	Rs	P
Weight	Length	0.98	<.0001
LSI	GSI	0.62	0.0025
LSI	Ugt	-0.77	0.0019

Table 3.11: Spearman's rank correlation results for males from the inner Oslofjord; variables, Rs: Spearman`s rank correlation coefficient and p-value.

Variable	Variable	Rs	P
Weight	Length	0.99	<.0001
Length	AChE activity	-0.70	0.001
Weight	AChE activity	-0.71	0.001
Condition	Vtg	0.85	0.0009

Table 3.12: Spearman's rank correlation results for females from the outer Oslofjord; variables, Rs: Spearman`s rank correlation coefficient and p-value.

Variable	Variable	Rs	P
Weight	Length	1	<.0001
OH - BaP	UGT	1	<.0001

Table 3.13: Spearman's rank correlation results for males from the outer Oslofjord; variables, Rs: Spearman`s rank correlation coefficient and p-value.

Variable	Variable	Rs	P
Weight	Length	0.90	<.0001
OH - Phen	HSP70	0.83	0.0017

3.5.1 Correlations between protein/enzyme and gene expression

The correlations between biomarkers measured at protein or enzyme activity level and gene expression as presented in table 3.14 to 3.17. None of the parameters had a significant correlation. Outer Oslofjord males had a near to significant correlation for MT gene expression and MT protein levels in liver.

Table 3.14: Spearman's rank correlation between CYP1A gene expression and EROD activity; Rs: Spearman`s rank correlation coefficient and p-value.

Group	Rs	P
Inner Oslofjord females	0.19	0.7
Inner Oslofjord males	0.64	0.1
Outer Oslofjord females	0.21	0.5
Outer Oslofjord males	0.17	0.7

Table 3.15: Spearman's rank correlation between UGT gene expression and EROD activity; Rs: Spearman`s rank correlation coefficient and p-value.

Group	Rs	P
Inner Oslofjord females	0.00	1
Inner Oslofjord males	0.14	0.8
Outer Oslofjord females	0.36	0.2
Outer Oslofjord males	0.23	0.5

Table 3.16: Spearman's rank correlation between MT gene expression and MT protein; Rs: Spearman`s rank correlation coefficient and p-value.

Group	Rs	P
Inner Oslofjord females	0.15	0.6
Inner Oslofjord males	0.43	0.2
Outer Oslofjord females	0.48	0.2
Outer Oslofjord males	0.58	0.06

Table 3.17: Spearman's rank correlation between HO-1 gene expression and MT protein; Rs: Spearman`s rank correlation coefficient and p-value.

Group	Rs	P
Inner Oslofjord females	0.00	1.0
Inner Oslofjord males	0.50	0.2
Outer Oslofjord females	-0.21	0.5
Outer Oslofjord males	-0.10	0.8

4 Discussion

4.1 Physiological indices

Based on otolith readings, cod from the outer Oslofjord had a maximum age of six years while the oldest cod individuals from the inner Oslofjord were four years old. Despite different maximum age, however, no significant difference in age distribution was apparent for cod collected from the two areas. Cod from the outer fjord had significantly larger size for the same age compared with the cod from the inner fjord, although with an exception of the three year olds which had no difference in size between the two areas. Cod has earlier been reported to grow faster in outer part of fjords (Gjøsæter et al., 2011) and this may also be true for cod in this study, but with exception from the three year olds. Other studies have also indicated that cod growth is highly variable both in nature and under lab conditions (Brander, 1995; Jobling, 1988) and cod growth is thought to be affected by factors like ambient water temperature and food consumption (Godø et al., 1987; Jørgensen, 1992). Suggestions have been made that cod will grow in a density-dependent manner (Stenseth et al., 1999) and this may therefore be the case for cod in a restricted area like the inner Oslofjord. There could also be other stressors in the inner Oslofjord than in the outer which may affect the growth, e.g. exposure to toxicants.

No differences were seen for condition factor between the two areas, although there was larger variability in the data for the outer Oslofjord. This implies that cod from both areas where in similar condition, but that some individuals in the outer Oslofjord had lower condition than cod from the inner Oslofjord. The condition factor is believed to be affected by several factors, but a low condition factor is generally thought to reflect low energy reserves (Azmat et al., 2007; Anderson et al., 1996; Lambert et al., 1997). Cod that is smaller than what is normal for its age have been suggested to also have reduced condition factor (Jobling et al., 1994). However, this was not the case for cod in the inner Oslofjord and the lower size than outer Oslofjord cod must have been due to other factors than nutrition in this area. For the LSI there were no differences between cod from the inner and outer Oslofjord. This is in accordance with the condition factor, since condition factor and LSI are measurements often used together to investigate the overall condition of fish (Dutil et al., 2003).

GSI was higher in females from the inner fjord compared with the outer fjord. This may indicate that females from the inner fjord have started the spawning process earlier than

females from the outer fjord. It has been observed that fish may decrease in size when they invest energy in reproduction (Thorpe, 1994) and since cod in the inner were generally smaller than cod from the outer fjord, this may be the reason. However, further analyses showed that the higher GSI trend in the inner fjord was due to the three-year old females which had a similar size at cod from the outer fjord. Therefore the smaller size in the inner fjord seemed not to be due to higher investment in gonadal development than cod from the outer fjord.

Other studies have also shown that cod from inner Oslofjord become sexually mature earlier than cod from other populations (Olsen et al., 2004). This might be due to local adaptations and different reproductive timing for the two populations as there is little migration between them. However, spawning time has also been suggested to be affected by differences in the environment rather than genetic differences (Godø et al., 1987). Other studies conducted in the inner Oslofjord did not observe a similar trend for GSI, but the whole body weight measurements were done without removing the intestine, which might explain the different results (Imrik, 2010; Holth, 2004). Therefore it cannot be concluded if the GSI have changed in recent years in inner Oslofjord females. But it is evident that the three year old females in the inner Oslofjord were more matured that the three year olds from the outer fjord. The same trend for higher GSI in the inner Oslofjord was also observed for males, but the difference was not statistically significant.

For the three year old males, LSI was significantly higher in the inner Oslofjord than in the outer fjord. This is in great contrast to the three year old females where the opposite pattern was found, although this was not statistically significant. LSI and GSI have been found to correlate negatively due to lipid transfer from liver to gonads with increased maturity of the fish (Lambert et al., 1997). But the three year old males from the inner Oslofjord had higher GSI than for outer fjord, although not significant, in addition to higher levels of LSI. This may be explained by that cod in the inner Oslofjord had sufficient nutrition available to maintain both high levels of LSI and GSI. This was not the case for females whereof the energy in the liver may have been used for gonad development.

In addition to the discussed factors affecting GSI, changes in GSI have also been used as a biomarker for exposure to estrogens (reviewed in Scholz et al., 2009). These results may therefore also be due to exposure to estrogens of inner Oslofjord cod as for example estradiol from wastewater. However, it is important to note that maturity and reproduction are complex

biological processes in fish and one should be careful when drawing conclusions (Arukwe et al., 1998)

4.2 Exposure to and effects of PAHs

The concentration of the PAH metabolite OH-phenanthrene in bile was not significantly different between cod sampled from the inner or outer Oslofjord. Fish are known to conjugate phenanthrene to more soluble metabolites such as OH-phenantrene in a dose dependent manner (Solbakken et al., 1981). This indicates that cod from the inner and outer Oslofjord examined in this study have been exposed to similar levels of phenanthrene. In 2002 there was found higher levels of OH-phenantrene in bile from inner Oslofjord cod than from outer Oslofjord cod (Holth, 2004). The level of OH-phenantrene was also higher in 2002 in inner Oslofjord than what was found in 2008 and in this study (Imrik, 2010). This indicates that cod has been exposed to lower amounts of phenantrene in recent years. Phenanthrene is a petrogene PAH found in fossil fuel (Krahn et al., 1986) and the reduced exposure may therefore be due to reduced concentrations of oil in the Oslofjord.

There were significantly higher concentrations of OH-pyrene in bile of cod from the inner compared with cod from the outer Oslofjord. This indicates that cod in the inner Oslofjord were exposed to higher levels of pyrene than cod from the outer Oslofjord. This is in accordance with findings from 2002 and 2008 (Holth 2004; Imrik 2010). Pyrene, as well as benzo(a)pyrene, are pyrogenic PAHs formed during incomplete combustion such as during burning of fossil fuel and from industrial burning activities (Manoli et al., 1999). This could indicate that industrial activity around the inner Oslofjord as well as the fossil fuel burning from e.g. boat traffic, were responsible for the major part of the total PAH released into the inner Oslofjord. The toxicity of pyrene is not well understood, but it is thought to be one of the carcinogenic PAHs, although less toxic than e.g. benzo(a)pyreneapyrene (Zeng et al., 2000). Within the inner Oslofjord, females had significantly higher levels of OH-pyrene than males. The reason for this is not clear, but it may be due to different behavior between female and male cod such as pelagic and bottom foraging (Fordham et al., 1999). This could have caused females and males to be exposed to different amounts of pyrene. Pyrene is a smaller PAH and it is known to be less bioavailable than the larger PAHs since it binds to soot particles (Hylland, 2006). This may cause pyrene to sediment and thereby cause bottom feeding fish to be more exposed.

In 2002 there was found high levels of both OH-pyrene and OH-phenenanthrene in inner Oslofjord compared to outer (Holth, 2004). However, in 2008 and in this study, only OH-pyrene was found to be higher in the inner fjord (Imrik, 2010). This shows that the relationships between these metabolites have changed during recent years and it may reflect changes in the sources of PAHs into the inner Oslofjord.

The concentrations of 3-OH–B(a)P in bile were not found to be significantly different between any of the groups tested, implying that fish from the two areas were exposed to similar concentration of B(a)P. In 2002, only 23% of the cod individuals from the Oslofjord had concentrations above detection limit (Holth, 2004). And in 2008 approximately 25% of cod from the Oslofjord had 3-OH-B(a)P concentrations above the detection limit (Imrik, 2010). In this study more than 40% of the individuals in every group had levels of 3-OH-B(a)P above the detection limit. The exposure to B(a)P in the Oslofjord may therefore have been increasing in recent years. B(a)P is known to be highly toxic to marine organisms by its ability to form DNA adducts (Van Veld et al., 1987). It is also one of the PAHs known to have highest affinity to the Ah-receptor and thereby induce expression of CYP1A (reviewed in Whyte et al., 2000).

There were no differences in hepatic EROD activity between the two areas, despite the higher hydrocarbon exposure in the inner compared with the outer Oslofjord found in this study. Similar results were also found in 2002 for this biomarker (Holth, 2004). However, in 2008 there was higher activity in hepatic EROD activity in cod from the inner Oslofjord compared with cod from the outer Oslofjord (Imrik, 2010). Many studies have observed elevated EROD activity when fish have been exposed to different toxicants such as planar PCBs, planar dioxins and PAHs (Ruus et al., 2002; Altenburger et al., 2003; Hektoen et al., 1994). But several internal and external factors are well known to inhibit the EROD activity, such as conditional level, ambient water temperature, maturity stage and oxygen deficiency (Andersson et al., 1992; Barker et al., 1994; Schnell et al., 2008). The handling of fish prior to analyses has also been suggested to decrease the enzyme activity (Lappivaara, 2001). Toxicants are also known to inhibit the EROD activity. Fish administrated to metals like copper and mercury was seen to have a decreased EROD activity and this was also the case for cadmium exposure which reduced the enzyme activity with as much as 90% (George et al., 1986; Viarengo et al., 1997). Organotin substances such as TBTs are also thought to inhibit the EROD activity at the protein/enzyme level, but not on the gene level (Brüschweiler et al., 1996). The possible inhibition of EROD activity found in the present study may have

the potential to alter the metabolism and thereby the elimination of some PAHs (Nacci et al., 2002). The toxicity of PAHs, such as the exposure to pyrene in the inner Oslofjord, may therefore be changed and can possibly cause it to be more toxic for cod in the inner Oslofjord. However, the low EROD activity could also be real due to low exposure to toxicants in the inner Oslofjord.

The gene expression of CYP1A was found to be up-regulated for both females and males in the inner Oslofjord in comparison with cod from the outer. CYP1a induction is a well-documented biomarker in fish for exposure to e.g. PAHs both on protein (e.g. Gadagbui et al., 1996) and gene level (e.g. Campbell et al., 1996). The elevated CYP1A transcription in inner Oslofjord cod may therefore be the result of the higher exposure to pyrene found in inner Oslofjord. However, the inner Oslofjord is also thought to be polluted with PCBs, TBTs and other toxicants that can induce the expression of CYP1A. As CYP1A was found to be up regulated in the inner Oslofjord, and no difference in EROD activity was found between the inner and outer fjord, this suggests that the EROD activity was inhibited in inner Oslofjord cod. This may be due to TBT exposure as this toxicant functions as an inducer of CYP1A gene expression, but as an inhibitor of EROD activity (Brüschweiler et al., 1996). However, this biomarker has been suggested to be a biomarker for the overall toxically load in organisms (Ewald, 1995) and the CYP1A induction may be due to exposure from other toxicants or mixtures as well.

There was also a significant difference in female and male expression of CYP1A. This is in accordance with the literature that females tend to have lower CYP1A activity than males when exposed to similar concentrations of e.g. PAHs (reviewed in Whyte et al., 2000). The different expression levels of CYP1A in females and males have been seen to increase even more in relation to spawning (Flammarion, 1999; Larsen et al., 1992) and fish administrated to estradiol show depressed expression of CYP1A (Gray et al., 1991). The estradiol receptor and the Ah-receptor are known to cross-talk (Lee et al., 2011) and the increased gonadal development in female fish from inner Oslofjord found in this study may contribute to the lower response for this group.

This variability often observed between sexes for some biomarkers has led to a discussion of whether female fish are less suitable for biomarker analyses, and considerations should be taken when interpreting the results for female fish (Goksøyr et al., 1997).

The gene expression level of uridine-diphosphate-glucuronosyltransferase (UGT) was not significantly different between cod from the inner and the outer Oslofjord. UGT can be

induced by exposure to e.g. PAHs (Williams et al., 2003) and the synthesis is regulated through binding to the same Ah–receptors as for CYP1A induction (Nelson et al., 1993). Therefore there have been found positive correlations for the up regulation for UGT and CYP1A (Williams et al., 2003). Therefore one could maybe expect to see a similar up-regulation of UGT as for CYP1A in this study. However, the synthesis of UGT may be less responsive than CYP1A in cod as has been found in other studies (Holth et al., 2010). Since UGT is less sensitive than CYP1A, this may indicate that the toxic load in the inner Oslofjord was not intense.

4.3 Effects from metal exposure

MT protein amount and MT mRNA expression are considered useful biomarkers for metal exposure in fish. In this study no differences in hepatic MT concentration for cod from the inner and outer Oslofjord, or between sexes, were found. This is different from 2002 where significantly lower MT concentrations were found in cod from the inner compared with the outer Oslofjord (Holth, 2004). In addition there was also observed higher concentrations of MT in female cod than in male. In 2008 there were no significant differences in MT concentration in liver between inner and outer Oslofjord, but there was found significantly higher concentrations in males than in females (Imrik, 2010). It has been shown that MT mRNA is a more sensitive biomarker than MT protein for metal exposure (Carginale et al., 1998; Van Cleef-Toedt et al., 2001). However, no significant differences in the relative gene expression of MT between cod from the inner and outer Oslofjord or between females and males were found in the present study.

HO-1 as well as MT, are considered to code for stress proteins as their synthesis increases in response to stressors (Lawrence et al., 2003). HO-1 is a well-known indicator for oxidative stress (Applegate et al., 1991; Lu et al., 2001) and metals can radiate metal ions which may become reactive oxygen species (Stohs et al., 1995). MT is in addition to binding metals, also thought to protect the cells from damage by free radicals (Olsson et al., 1995). There were no differences in HO-1 gene expression in cod from inner or outer Oslofjord. Although there was some larger variability in the expression for males from the inner Oslofjord compared with the other groups. Based on HO-1 expression and MT gene expression and protein, it may seem that cod from the inner and outer Oslofjord had been exposed to similar amounts of metals.

It is nevertheless important to monitor the sea carefully for metal exposure based on the increased concentration of mercury found in freshwater fish recent years (Fjeld et al., 2009) and the increased tendency towards higher content of some metals in cod from the inner Oslofjord (Green et al., 2010b). One must also take into consideration other factors affecting the MT content such as the female reproduction cycle. Since the hepatic MT concentration is known to decrease in females during the spawning process (Olsson et al., 1989), this may affect the MT content in female cod in the inner Oslofjord as they showed higher GSI than outer Oslofjord females.

ALA-D is considered a very specific marker for inhibition from lead exposure, and it is also considered to be a sensitive method. There were no significant differences in ALA-D activity between cod from inner and outer Oslofjord, or between the sexes, indicating that cod in the two areas are exposed to similar levels of lead. For males there was a higher variability than for females from both areas and some individuals in the outer fjord seemed to have higher activity of ALA-D than individuals from the inner fjord. In 2002, the activity of ALA-D was observed to be lower in cod from the inner compared with the outer fjord and there was also observed difference in enzyme activity between the sexes (Holth, 2004). In 2008 there was also observed lower activity of ALA-D in the inner Oslofjord, but no differences for sex (Imrik, 2010). Both these studies showed higher levels of lead exposure than in the present study. The similar activity of ALA-D indicates that the levels of lead exposure to cod in the Oslofjord have decreased and this was also suggested in Green et al. (2010).

4.4 Effects from toxicants inhibiting AChE activity

The results from the measurements of AChE activity in muscle homogenate, showed a significantly lower activity in females from outer Oslofjord than females from inner Oslofjord. For males there were no differences, although some individuals from the outer fjord seemed to have lower AChE activity. This indicates that cod from the outer Oslofjord have been exposed to toxicants that inhibit the AChE enzyme activity. This is similar to what was found for cod in the Oslofjord in 2008 (Imrik, 2010). Several substances inhibit AChE, but pesticides such as organophosphates and carbamates are among the most potent inhibitors (Olson et al., 1980; Payne et al., 1996). There are several possible sources of pesticides to the outer Oslofjord. For instance farmlands, which are common around the outer Oslofjord, may

be a source of pesticides. But also other sources such as formulating plants and manufacturing processes are known to produce pesticides (Parveen et al., 2005).

The toxicants inhibiting AChE are considered to be neurotoxins, but fish have been shown to quickly recover from inhibited enzyme activity (Bhattacharya, 2001). This can indicate that a chronic exposure is harmful, but if the exposure is acute the fish may be able to deal with this stressor. If the effect observed in the outer Oslofjord is due to exposure to pesticides from farmlands, the exposure can be expected to occur in an acute manner with peaks before or during the growing season and after heavy rainfall (Kreuger, 1998). However, since cod were sampled in November and because organophosphates rapidly degrades in the environment (Fulton et al., 2001) pesticides might not be the reason for the observed inhibition of AChE. Metals can also inhibit AChE activity (Olson et al., 1980) but as there were no significant exposure for outer Oslofjord seen in the biomarkers for metal exposure this seems unlikely. It is possible that the industry located around the outer Oslofjord contributes with toxicants that can inhibit AChE activity. Other sources of contaminants to the outer Oslofjord may be input from rivers and waterways such as the Drammen River (Ludvigsen, 2005). Nevertheless, cod from the outer Oslofjord had a similar inhibition in the AChE enzyme activity as in 2008 (Imrik, 2010), which shows that the cod is still exposed to inhibitory toxicants.

4.5 Vtg and hsp70 gene expression

Vtg is a well-known biomarker for exposure to environmental estrogens in males (e.g. García-Reyero et al., 2004; Mellanen et al., 1999). There were found no differences in gene expression of Vtg in cod from the inner and outer Oslofjord. However, males in the inner fjord did show increased expression of Vtg (expression factor 5.44) compared to males from the outer fjord. This indicates that some male individuals from the inner Oslofjord have been exposed to estrogens. Measurements of Vtg protein concentration in 2002 of inner Oslofjord males, showed no differences in comparison with males from the outer Oslofjord (Holth, 2004). In 2006 however, elevated levels were seen in inner Oslofjord male cod (Scott et al., 2006). Wastewater treatment plants are known to contribute with estrogens such as the birth control rethinylestradiol (Thorpe et al., 2003). Since studies have showed that even very low concentrations (ng/L) may cause feminisation of male fish (Metcalfe et al., 2001) this can be thought to be the reason for the high Vtg expression observed in some inner Oslofjord males.

The tendency towards higher GSI for inner Oslofjord cod and the increased Vtg in some males from this area might be biologically relevant. One can speculate whether both the Vtg results and the higher GSI in inner Oslofjord cod were due to exposure to toxicants. There were no differences in Vtg expression between females from the two areas. This is in contrast to the GSI levels which showed a higher maturity status for inner Oslofjord females compared with females form the outer fjord. The reason why these two biomarkers differ in female cod is unknown, but it may be due to the different repose time for the different organisational levels measured. For the expression of hsp70 there were no significant differences between areas or sexes. This indicates that none of the populations had been differently exposed to stressors like e.g. heat stress, exposure to metals or other toxicants that are known to cause induction of this gene.

4.6 Correlations

In the inner Oslofjord, females with high liver weight in comparison to the somatic body weight also had higher gonadal development. This is seen from the positive correlation between LSI and GSI. Fish exposed to the estrogen 17-β-estradiol have been shown to have increased LSI (Åkerblom et al., 2000). During oocyte growth, Vtg is produced in the liver and transported to the ovaries, where it is incorporated into the oocytes. Estrogens are known to impact this formation of Vtg and can thereby cause a positive correlation between LSI and GSI even when out of season (Mommsen et al., 1988). As discussed earlier, this may also indicate that the fish had sufficient energy levels to maintain both high LSI and GSI.

There was also a negative correlation between LSI and UGT for inner Oslofjord females. This shows that female cod from the inner fjord that had high expression of UGT also had low LSI. There are no clear links between these two factors. But it may show that female fish in the inner Oslofjord had been exposed to toxicants that induce UGT and at the same time cause damages to the liver such as cell death.

For males in the inner Oslofjord there were significant negative correlations between AChE activity and both length and weight. This shows that larger sized males had more inhibited AChE activity than smaller males in this group. Other studies have also found that AChE activity was inversely proportional with the length of the fish (Burgeot et al., 1996). This can be explained by an increased bioconcentration of toxicants such as pesticides with increased body length (Katagi, 2010). There was also a positive correlation between LSI and

Vtg for males from the inner Oslofjord. In this group, there was also a tendency towards higher GSI for this group in comparison with the outer Oslofjord. This suggests that Vtg production in the liver may have been triggered by estrogen exposure, which may resulted in higher LSI.

3-OH-B(a)P is known to have high affinity to the Ah-receptor (Hylland, 2006) and this is highly evident in this study because of the complete positive correlation for B(a)P exposure and UGT expression in outer Oslofjord females. PAH exposure has previously been seen to induce UGT expression in fish (Williams et al., 2003) This complete correlation may imply that UGT expression is a sensitive measure for 3-OH-B(a)P exposure in cod even for small concentrations, at least based on the correlation seen in this group.

OH-phenantrene concentration in bile of male cod from the outer Oslofjord was found to correlate positively with hsp70. This can indicate an increased stress protein induction for the individuals in this group that were exposed to the higher levels of phenanthrene. As discussed earlier, exposure to toxicants can cause induction of hsp70 transcription and may be the reason for this correlation. Although to my knowledge, no studies have seen this specific link in fish.

In this study biomarkers at several organisational levels have been investigated. The correlation analyses for these levels revealed no significant correlations between biomarkers for protein concentration/enzyme activity and gene expression for any of the groups. Although a near to significant correlation for MT gene expression and MT protein concentration in outer Oslofjord males was found. Overall, this shows that biomarkers measured at different organisational levels in this study did not show similar response from exposure to toxicants. There could be several reasons for this as biomarkers are affected by many different factors. Gene expression may be more sensitive for lower concentrations of toxicants, but on the other hand, the response has a shorter half-life than for proteins and enzymes. Alteration in enzyme activity as a consequence of exposure to toxicants may last longer and thereby be measurable even after ended exposure. However, enzyme activity may also be inhibited by several internal and external factors.

4.7 Conclusions

Several parameters in this study have revealed significant differences between cod from the inner and outer Oslofjord that may reveal different exposure to toxicants. There was a trend towards smaller size and higher GSI in the inner Oslofjord, except for the three-year olds which seemed to be equal in size in comparison to cod from the outer Oslofjord, but with a higher GSI. This may indicate that cod in the inner Oslofjord were more mature than cod from the outer Oslofjord. The fact that there generally were no significant differences in condition factor and LSI between the two populations may indicate that there were other factors, like enhanced nutritional status that caused this difference in maturity, and it may be speculated whether if this was due to exposure to endocrines.

Cod from the inner Oslofjord may also have been exposed to higher concentrations of pyrene based on the metabolites in their bile. The trend from recent years indicates that oil pollution contributes with less PAHs to the inner Oslofjord, while the industry and burning of fossil fuel may have contributed to a higher extent.

Biomarkers for effects revealed that EROD activity might have been inhibited in the inner Oslofjord in comparison with earlier years. The higher expression of CYP1A in inner Oslofjord cod is also an indication of this. This may be due to exposure to TBTs since this group has the potential to both induce CYP1A and at the same time inhibit EROD activity. This is also based on the chemical measurements done in the inner fjord, which revealed detectable concentrations of TBT. For metal, no significant differences in exposure could be detected. This may indicate that the two populations were exposed to similar amounts of metals.

Cod from the outer Oslofjord had been exposed to inhibitory toxicants of AChE activity but which substances are unclear. This is unlikely to be caused by pesticide exposure, but it could be due to releases of metals or other industrial waste to the outer Oslofjord. These substances seem to bioaccumulate in male cod from the inner Oslofjord based on the correlation analyses.

Vtg measurements of males revealed that some males in the inner fjord might have been exposed to estrogens from e.g. wastewater. This may be seen in connection with the elevated levels of GSI. If this is the case this could have the potential to cause a change in the reproductive pattern of cod in the inner Oslofjord, which can ultimately have severe effects on the population.

Based on this study, both gene expression and protein/enzyme levels can contribute to the overall knowledge about the effects caused by toxicants in fish. They did not show the same picture, but together they may provide powerful analyses that can give highly important information about the effects of exposure for a range of toxicants in fish. Physiological indices will also contribute with useful information about the condition of the fish individuals and effects seen on this level will be of high biological relevancy, such as alteration in GSI for some age groups in the inner Oslofjord. It is also important to monitor the condition of the fish because individuals with lower condition can be more susceptible for different stressors such as toxicant exposure.

4.8 Future perspectives

Since the two cod populations are considered to be relatively isolated, and maybe also genetically different, it will be interesting to do a "common garden" experiment where both populations are exposed to controlled concentrations of different toxicants. Would the biomarkers reveal similar response? Cod from the inner Oslofjord may be less sensitive to exposure since they live in an environment that are believed to be more polluted than outer Oslofjord cod and may therefore have adapted to this environment. It will also have be interesting to investigate the exposure of estrogens further. Since even very small concentrations are thought to cause hormonal disruptions and eventually lead to reproduction failure, this is of high importance for the cod population in the inner Oslofjord.

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6 Appendix

Data material

Physiological parameters for cod from inner and outer Oslofjord

CF: condition factor, LSI: liver somatic index, GSI: gonadal somatic index

Code	Area	Sex	Length (cm)	Carcass (g)	Liver (g)	Gonad (g)	Age	CF	LSI	GSI
09i1	Inner	♂	47	733	9	3	3	0.84	1.17	0.40
09i2	Inner	♀	46	731	23	21	4+	0.88	3.09	2.72
09i3	Inner	♀	37	430	5	1	3	0.92	1.06	0.32
09i4	Inner	♀	56	1498	32	8	3	0.94	2.07	0.51
09i5	Inner	♂	40	527	19	11	2+	0.98	3.39	1.96
09i6	Inner	♀	54	1231	34	30	2	0.90	2.65	2.33
09i7	Inner	♀	46	706	14	26	3	0.84	1.89	3.51
09i8	Inner	♂	45	708	8	1	4	0.91	1.05	0.08
09i9	Inner	♂	52	1024	16	29	3+	0.82	1.52	2.70
09i10	Inner	♀	49	985	29	21	2+	0.98	2.86	2.05
09i11	Inner	♀	58	1397	13	10	3+	0.79	0.90	0.67
09i12	Inner	♀	52	926	40	18	2+	0.82	4.16	1.81
09i13	Inner	♂	57	1835	34	2	3	1.10	1.80	0.09
09i14	Inner	♀	49	909	23	14	3	0.87	2.49	1.46
09i15	Inner	♀	41	547	18	13	2+	0.92	3.22	2.28
09i16	Inner	♀	51	938	15	19	3	0.80	1.60	1.96
09i17	Inner	♂	44	591	13	3	3	0.79	2.09	0.53
09i18	Inner	♀	52	1619	47	29	3+	1.27	2.84	1.72
09i19	Inner	♂	55	1475	41	117	3	1.03	2.67	7.17
09i20	Inner	♀	43	689	10	4	3	0.95	1.45	0.57
09i21	Inner	♂	53	1279	19	1	2+	0.97	1.48	0.08
09i22	Inner	♂	47	811	34	19	3	0.97	4.06	2.24
09i23	Inner	♀	41	615	13	3	3	1.01	2.05	0.40
09i24	Inner	♂	38	469	10	0	3+	0.98	1.99	0.08
09i25	Inner	♂	65	2577	26	3	3	1.00	1.01	0.10
09i26	Inner	♀	52	988	18	17	4	0.92	1.79	1.70
09i27	Inner	♂	59	1692	66	50	3	0.95	3.77	2.77
09i28	Inner	♀	45	612	11	15	3+	0.81	1.77	2.32
09i29	Inner	♀	40	448	6	2	1+	0.84	1.23	0.37
09i30	Inner	♀	39	456	5	2	2+	0.84	1.00	0.37
09i31	Inner	♀	43	642	6	2	2+	0.92	0.94	0.31
09i32	Inner	♂	38	440	5	0	4	0.92	1.01	0.02

09i33	Inner	♀	41	570	12	13	2	0.97	2.10	2.10
09i34	Inner	♂	39	471	9	23	2+	0.94	1.94	4.53
09i35	Inner	♂	49	856	18	10	2	0.86	2.10	1.07
09i36	Inner	♂	42	669	11	0	3	1.06	1.56	0.01
09i37	Inner	♂	59	1845	75	100	3	1.06	3.89	4.93
09i38	Inner	♀	41	543	10	10	4+	0.86	1.79	1.74
09i39	Inner	♂	35	351	4	0	2+	0.91	1.15	0.06
09i40	Inner	♂	37	403	10	1	4	0.94	2.49	0.29
09Y1	Outer	♂	49	1012	20	59	2+	1.05	1.95	5.43
09Y2	Outer	♀	55	1435	41	4	5+	0.95	2.79	0.30
09Y3	Outer	♂	58	1485	43	1	2	0.85	2.83	0.07
09Y4	Outer	♂	47	908	14	13	3	0.96	1.52	1.34
09Y5	Outer	♀	44	664	11	1	3	0.85	1.69	0.21
09Y6	Outer	♂	49	930	13	28	5+	0.87	1.40	2.83
09Y7	Outer	♂	57	768	14	1	5+	0.53	1.73	0.06
09Y8	Outer	♀	53	1467	59	87	2+	1.13	3.84	5.37
09Y9	Outer	♀	54	1387	38	28	4	1.07	2.63	1.92
09Y10	Outer	♀	40	445	6	2	3	0.75	1.33	0.44
09Y11	Outer	♂	56	1516	20	21	4+	0.96	1.27	1.32
09Y12	Outer	♀	48	895	20	2	4	0.92	2.13	0.23
09Y13	Outer	♀	49	1004	24	8	4	0.95	2.33	0.80
09Y14	Outer	♂	46	858	20	1	6	0.95	2.25	0.15
09Y15	Outer	♂	43	665	15	5	2+	0.92	2.26	0.72
09Y16	Outer	♂	45	732	6	0	3	0.88	0.80	0.03
09Y17	Outer	♂	51	1495	14	1	3	1.18	0.92	0.05
09Y18	Outer	♀	48	971	30	5	5	0.99	3.01	0.48
09Y19	Outer	♀	55	1532	38	15	4	1.03	2.42	0.95
09Y20	Outer	♀	52	1258	5	9	2	0.91	0.37	0.71
09Y21	Outer	♀	51	1131	15	6	3	0.95	1.29	0.52
09Y22	Outer	♀	52	1159	9	4	4	0.91	0.77	0.34
09Y23	Outer	♀	57	1267	27	9	4	0.74	2.09	0.66
09Y24	Outer	♀	54	640	8	2	3+	0.46	1.22	0.31
09Y25	Outer	♀	40	526	10	6	4+	0.92	1.83	1.14
09Y26	Outer	♀	52	1065	22	4	6	0.83	2.00	0.39
09Y27	Outer	♀	59	1590	74	6	3+	0.82	4.45	0.37
09Y28	Outer	♀	57	1407	15	13	1+	0.85	1.07	0.93
09Y29	Outer	♂	52	1281	21	6	3+	0.98	1.60	0.47
09Y30	Outer	♀	52	1188	23	14	2	0.99	1.93	1.11
09Y31	Outer	♀	50	1019	21	5	1	0.88	1.99	0.46
09Y32	Outer	♂	60	1815	25	1	2+	0.93	1.38	0.05
09Y33	Outer	♀	43	612	22	4	3+	0.90	3.50	0.69
09Y34	Outer	♀	41	556	20	2	3	0.89	3.44	0.29
09Y35	Outer	♂	44	692	21	9	4+	0.94	3.00	1.19

09Y36	Outer	♂	47	833	15	5	3+	0.89	1.71	0.57
09Y37	Outer	♀	46	902	19	18	5	1.04	2.09	1.87
09Y38	Outer	♀	51	1098	62	10	2	0.96	5.31	0.89
09Y39	Outer	♂	44	714	26	3	1+	0.91	3.49	0.35
09Y40	Outer	♂	50	1086	35	37	2+	1.00	3.14	3.16

Biomarkers data for cod from inner and outer Oslofjord

Code	Area	Sex	OH-Phen	OH-pyrene	3-OH-B(a)p	ALA-D	AChE	EROD	MT
09i1	Inner	♂	3.6	152	NA	17	4.4	2	16
09i2	Inner	♀	6.7	154	NA	17	2.5	15	21
09i3	Inner	♀	10.2	190	NA	11	4.5	NA	10
09i4	Inner	♀	19.5	108	4.4	18	3.6	NA	15
09i5	Inner	♂	13.1	194	3.5	9	5.5	90	NA
09i6	Inner	♀	3.4	332	2.0	23	4.0	69	16
09i7	Inner	♀	7.3	238	2.2	12	4.5	NA	6
09i8	Inner	♂	NA	135	NA	16	4.1	NA	17
09i9	Inner	♂	4.3	210	3.2	18	4.8	0	33
09i10	Inner	♀	13.7	372	NA	17	2.4	30	NA
09i11	Inner	♀	6.8	242	NA	12	5.1	NA	29
09i12	Inner	♀	10.4	456	2.2	16	2.7	0	15
09i13	Inner	♂	8.5	257	NA	14	2.1	24	NA
09i14	Inner	♀	0.0	248	NA	12	5.4	NA	8
09i15	Inner	♀	6.0	266	NA	20	3.9	54	9
09i16	Inner	♀	7.6	108	2.1	15	4.5	0	36
09i17	Inner	♂	12.7	147	NA	50	4.4	2	14
09i18	Inner	♀	9.1	241	NA	NA	2.8	NA	NA
09i19	Inner	♂	9.8	290	NA	13	1.6	NA	13
09i20	Inner	♀	3.8	310	NA	13	9.4	0	9
09i21	Inner	♂	9.1	84	NA	19	3.5	46	16
09i22	Inner	♂	5.6	187	2.0	10	2.2	94	19
09i23	Inner	♀	NA	323	2.4	12	3.4	107	NA
09i24	Inner	♂	8.8	192	2.1	18	3.8	0	24
09i25	Inner	♂	9.6	274	NA	17	2.3	NA	32
09i26	Inner	♀	30.4	221	3.3	14	4.1	10	17
09i27	Inner	♂	17.3	349	2.6	57	1.6	NA	6
09i28	Inner	♀	NA	162	3.0	18	4.5	17	27
09i29	Inner	♀	15.6	246	NA	13	4.8	81	19
09i30	Inner	♀	5.0	151	NA	14	3.8	NA	25
09i31	Inner	♀	27.3	269	NA	16	8.7	93	11
09i32	Inner	♂	17.2	141	3.5	5	6.8	37	16
09i33	Inner	♀	9.6	206	NA	13	4.6	144	31

09i34	Inner	♂	4.5	143	NA	8	4.9	98	20
09i35	Inner	♂	11.1	219	2.2	15	8.0	NA	35
09i36	Inner	♂	18.4	103	3.0	14	2.7	NA	15
09i37	Inner	♂	9.2	128	2.2	11	1.7	NA	28
09i38	Inner	♀	15.5	421	4.0	16	4.2	NA	13
09i39	Inner	♂	7.3	363	NA	22	4.2	NA	26
09i40	Inner	♂	5.9	121	2.3	16	4.7	120	18
09Y1	Outer	♂	19.2	19	2.8	19	4.2	0.4	21
09Y2	Outer	♀	2.3	33	3.5	19	2.4	18	11
09Y3	Outer	♂	9.3	20	NA	21	3.2	NA	NA
09Y4	Outer	♂	15.4	46	NA	50	3.5	NA	7
09Y5	Outer	♀	5.1	24	NA	10	3.5	59	18
09Y6	Outer	♂	27.7	28	4.3	21	3.5	NA	9
09Y7	Outer	♂	6.0	32	NA	24	1.9	12	5
09Y8	Outer	♀	5.3	34	NA	22	2.1	NA	19
09Y9	Outer	♀	NA	12	NA	25	3.1	37	17
09Y10	Outer	♀	9.1	20	NA	17	3.0	0	NA
09Y11	Outer	♂	8.6	25	NA	12	2.7	2	12
09Y12	Outer	♀	NA	30	NA	21	3.8	NA	20
09Y13	Outer	♀	11.1	31	NA	8	2.4	NA	21
09Y14	Outer	♂	21.6	62	3.8	35	2.7	54	11
09Y15	Outer	♂	14.1	53	3.2	12	3.7	91	13
09Y16	Outer	♂	14.7	31	NA	15	5.5	37	33
09Y17	Outer	♂	7.6	23	NA	25	2.7	2	15
09Y18	Outer	♀	4.3	24	3.8	15	4.6	20	32
09Y19	Outer	♀	6.2	38	NA	13	4.9	101	22
09Y20	Outer	♀	16.7	21	NA	20	2.1	0	15
09Y21	Outer	♀	8.8	53	NA	13	3.4	94	26
09Y22	Outer	♀	NA	27	NA	13	2.2	73	15
09Y23	Outer	♀	25.1	38	NA	15	3.2	39	15
09Y24	Outer	♀	14.9	32	2.5	14	4.4	NA	23
09Y25	Outer	♀	3.0	32	2.3	15	4.1	NA	6
09Y26	Outer	♀	20.5	29	2.5	16	2.2	53	5
09Y27	Outer	♀	20.8	19	3.1	23	2.8	0	11
09Y28	Outer	♀	7.7	29	NA	18	4.2	25	29
09Y29	Outer	♂	3.4	19	NA	13	4.0	50	13
09Y30	Outer	♀	4.8	23	NA	14	7.5	0	30
09Y31	Outer	♀	23.2	35	2.3	28	4.0	75	9
09Y32	Outer	♂	7.3	34	NA	14	2.0	78	9
09Y33	Outer	♀	3.6	36	5.6	21	2.7	20	33
09Y34	Outer	♀	5.6	46	2.5	12	4.2	14	5
09Y35	Outer	♂	16.4	54	NA	15	1.9	27	16
09Y36	Outer	♂	5.0	37	2.2	12	2.2	105	12
09Y37	Outer	♀	12.5	17	2.5	22	4.0	9	19
09Y38	Outer	♀	27.6	18	5.1	15	4.1	NA	2
09Y39	Outer	♂	27.8	195	3.3	33	3.5	14	7

09Y40	Outer	♂	NA	97	2.4	13	3.1	34	NA

OH-Phenantrene, OH-pyrene and OH-Benzo(a)pyrene; ng/g, ALA-D activity; ng PBG/min/mg protein, AChE activity; nmol/min/mg protein, EROD activity; pmol/min/mg protein, MT concentration; µg/mg protein

Relative Gene expression for cod from inner and outer Oslofjord

See table 2.5 for complete gene names.

Code	Area	Sex	ACTB	CYP1a	MT	HSP70	HO-1	Vtg	UGT
09i1	Inner	♂	0.0425	0.0526	0.0495	0.0329	0.0457	0.0309	0.0403
09i3	Inner	♀	0.0422	0.0529	0.0498	0.0332	0.0450	0.0372	0.0404
09i5	Inner	♂	0.0424	0.0522	0.0522	0.0323	0.0408	0.0500	0.0409
09i7	Inner	♀	0.0428	0.0506	0.0480	0.0352	0.0388	0.0337	0.0425
09i8	Inner	♂	0.0421	0.0509	0.0510	0.0323	0.0436	0.0524	0.0394
09i11	Inner	♀	0.0419	0.0527	0.0517	0.0329	0.0427	0.0438	0.0413
09i12	Inner	♀	0.0422	0.0476	0.0507	0.0332	0.0415	0.0610	0.0402
09i13	Inner	♂	0.0421	0.0497	0.0500	0.0334	0.0449	0.0329	0.0408
09i15	Inner	♀	0.0422	0.0485	0.0453	0.0332	0.0384	0.0458	0.0406
09i16	Inner	♀	0.0416	0.0479	0.0496	0.0327	0.0403	0.0377	0.0389
09i18	Inner	♀	0.0423	0.0528	0.0487	0.0337	0.0463	0.0407	0.0412
09i19	Inner	♂	0.0438	0.0531	0.0486	0.0331	0.0373	0.0417	0.0435
09i21	Inner	♂	0.0420	0.0493	0.0477	0.0339	0.0451	0.0375	0.0406
09i24	Inner	♂	0.0428	0.0538	0.0486	0.0332	0.0438	0.0287	0.0413
09i25	Inner	♂	0.0413	0.0526	0.0534	0.0333	0.0460	0.0288	0.0400
09i26	Inner	♀	0.0424	0.0525	0.0511	0.0323	0.0472	0.0463	0.0417
09i28	Inner	♀	0.0404	0.0506	0.0509	0.0340	0.0484	0.0417	0.0406
09i29	Inner	♀	0.0428	0.0506	0.0486	0.0325	0.0462	0.0340	0.0407
09i32	Inner	♂	0.0416	0.0516	0.0503	0.0334	0.0484	0.0309	0.0396
09i34	Inner	♂	0.0419	0.0523	0.0477	0.0335	0.0445	0.0333	0.0386
09i37	Inner	♂	0.0438	0.0570	0.0505	0.0320	0.0311	0.0367	0.0432
09i38	Inner	♀	0.0416	0.0507	0.0525	0.0326	0.0423	0.0485	0.0413
09i39	Inner	♂	0.0423	0.0518	0.0496	0.0340	0.0454	0.0294	0.0423
09i40	Inner	♂	0.0441	0.0567	0.0514	0.0330	0.0417	0.0377	0.0405
09Y2	Outer	♀	0.0422	0.0468	0.0499	0.0337	0.0461	0.0312	0.0398
09Y5	Outer	♀	0.0429	0.0497	0.0484	0.0333	0.0421	0.0419	0.0423
09Y6	Outer	♂	0.0413	0.0480	0.0483	0.0322	0.0390	0.0331	0.0410
09Y8	Outer	♀	0.0454	0.0491	0.0494	0.0330	0.0456	0.0300	0.0410
09Y10	Outer	♀	0.0429	0.0487	0.0496	0.0332	0.0451	0.0399	0.0416
09Y12	Outer	♀	0.0429	0.0485	0.0486	0.0328	0.0361	0.0451	0.0431
09Y13	Outer	♀	0.0416	0.0479	0.0490	0.0333	0.0452	0.0365	0.0408
09Y17	Outer	♂	0.0432	0.0450	0.0496	0.0329	0.0423	0.0484	0.0407
09Y18	Outer	♀	0.0411	0.0480	0.0520	0.0350	0.0486	0.0404	0.0431
09Y19	Outer	♀	0.0434	0.0540	0.0484	0.0325	0.0464	0.0485	0.0414
09Y21	Outer	♀	0.0432	0.0469	0.0507	0.0334	0.0455	0.0573	0.0415

09Y22	Outer	♀	0.0438	0.0479	0.0517	0.0330	0.0479	0.0424	0.0395
09Y23	Outer	♀	0.0409	0.0457	0.0503	0.0331	0.0430	0.0726	0.0399
09Y25	Outer	♀	0.0439	0.0464	0.0455	0.0339	0.0396	0.0308	0.0400
09Y27	Outer	♀	0.0420	0.0477	0.0509	0.0331	0.0447	0.0310	0.0411
09Y28	Outer	♀	0.0419	0.0461	0.0511	0.0334	0.0458	0.0313	0.0389
09Y29	Outer	♂	0.0420	0.0483	0.0493	0.0328	0.0432	0.0315	0.0416
09Y30	Outer	♀	0.0397	0.0491	0.0513	0.0329	0.0472	0.0338	0.0406
09Y34	Outer	♀	0.0418	0.0512	0.0497	0.0335	0.0458	0.0298	0.0421
09Y35	Outer	♂	0.0438	0.0523	0.0466	0.0326	0.0428	0.0382	0.0424
09Y36	Outer	♂	0.0407	0.0473	0.0501	0.0329	0.0405	0.0359	0.0421
09Y37	Outer	♀	0.0433	0.0475	0.0479	0.0343	0.0431	0.0311	0.0416
09Y38	Outer	♀	0.0427	0.0499	0.0493	0.0327	0.0461	0.0330	0.0401
09Y39	Outer	♂	0.0424	0.0476	0.0532	0.0339	0.0456	0.0305	0.0409
09Y40	Outer	♂	NA	NA	NA	NA	NA	NA	NA

All genes are presented as 1/Cp.

Plate layouts for EROD, ALA-D and AChE assay

EROD activity

S:sample, RS: Resorufin standard, REF: reference sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	RS 1	RS 2	RS 3	RS 4	RS 5	RS 6	RS 7	RS 8	BLANK	BLANK	BLANK	BLANK
B	RS 1	RS 2	RS 3	RS 4	RS 5	RS 6	RS 7	RS 8	BLANK	BLANK	BLANK	BLANK
C	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	Ref
D	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	Ref
E	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	Ref
F	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	Ref
G	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	Ref
H	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	Ref

+ RS 2

ALA-D activity

S:sample, PBG: Porphobilinogen standard, REF: reference sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBG std 1	PBG std 1	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	Ref
B	PBG std 2	PBG std 2	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	Ref
C	PBG std 3	PBG std 3	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	Ref
D	PBG std 4	PBG std 4	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	Ref
E	PBG std 5	PBG std 5	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	Ref
F	PBG std 6	PBG std 6	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	Ref
G	BLANK	BLANK	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	Ref
H	BLANK	BLANK	S 1	S 2	S 3	S 4	S 5	S 6	S 7	S 8	S 9	Ref

+ ALA

AChE activity

S:sample, REF: Reference sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	S2	S4	S6	S8	S10	S12	S14	S16	S18	S20	S22
B	Blank	S2	S4	S6	S8	S10	S12	S14	S16	S18	S20	S22
C	Blank	S2	S4	S6	S8	S10	S12	S14	S16	S18	S20	S22
D	Blank	S2	S4	S6	S8	S10	S12	S14	S16	S18	S20	S22
E	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	S21	REF
F	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	S21	REF
G	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	S21	REF
H	S1	S3	S5	S7	S9	S11	S13	S15	S17	S19	S21	REF

RT-qPCR

cDNA plate 1; std: standard, S:sample, nec: no enzyme control, ntc: no template control

	1	2	3	4	5	6	7	8	9	10	11	12
A	std 400 ng	std 400 ng	std 400 ng	Std 200 ng	Std 200 ng	Std 200 ng	Std 100 ng	Std 100 ng	Std 100 ng	Std 50 ng	Std 50 ng	Std 50 ng
B	Std 25 ng	Std 25 ng	Std 25 ng	S1	S1	S1	S2	S2	S2	S3	S3	S3
C	S4	S4	S4	S5	S5	S5	S6	S6	S6	S7	S7	S7
D	8	8	8	9	9	9	10	10	10	11	11	11
E	S12	S12	S12	S13	S13	S13	S14	S14	S14	S15	S15	S15
F	S16	S16	S16	S17	S17	S17	S18	S18	S18	S19	S19	S19
G	S20	S20	S20	S21	S21	S21	S22	S22	S22	nec	nec	nec
H	S23	S23	S23	S24	S24	S24	S25	S25	S25	ntc	ntc	ntc

cDNA plate 2; S:sample, nec: no enzyme control, ntc: no template control

	1	2	3	4	5	6	7	8	9	10	11	12
A	S26	S26	S26	S27	S27	S27	S28	S28	S28	S29	S29	S29
B	S30	S30	S30	S31	S31	S31	S32	S32	S32	S33	S33	S33
C	S34	S34	S34	S35	S35	S35	S36	S36	S36	S37	S37	S37
D	S38	S38	S38	S39	S39	S39	S40	S40	S40	S41	S41	S41
E	S42	S42	S42	S43	S43	S43	S44	S44	S44	S45	S45	S45
F	S46	S46	S46	S47	S47	S47	S48	S48	S48	S49	S49	S49
G	S50	S50	S50							nec	nec	nec
H										ntc	ntc	ntc

Correlation

Significant correlations with $p<0.05$.

Spearman's rank correlation for females from the inner Oslofjord; variables, Rs: Spearman`s rank correlation coefficient and p– value.

Variable	Variable	Rs	P
Weight	Length	0.98	<.0001
LSI	GSI	0.62	0.003
Age	EROD activity	- 0.61	0.04
Length	EROD activity	- 0.61	0.05
LSI	Ugt	-0.77	0.002
LSI	AChE activity	-0.61	0.005
GSI	HSP70	-0.64	0.01
OH - Pyrene	MT protein	-0.49	0.04
OH - BaP	OH-Phen	0.86	0.01

Spearman's rank correlation results for males from the inner Oslofjord; variables, Rs: Spearman`s rank correlation coefficient and p– value.

Variable	Variable	Rs	P
Weight	Length	0.99	<.0001
LSI	GSI	0.65	0.007
Length	AChE activity	-0.70	0.001
Weight	AChE activity	-0.71	0.001
Condition	AChE activity	- 0.62	0.006
Condition	Vtg	0.85	0.0009
LSI	EROD activity	0.68	0.04
LSI	Vtg	0.79	0.004
LSI	MT mRNA	0.74	0.01
OH - pyrene	HSP70	0.75	0.009
Vtg	AChE	- 0.64	0.04

Spearman's rank correlation results for females from the outer Oslofjord; variables, Rs: Spearman`s rank correlation coefficient and p– value.

Variable	Variable	Rs	P
Weight	Length	1	<.0001
OH - BaP	UGT	1	<.0001
GSI	Condition	0.55	0.008

LSI	EROD	-0.52	0.04
LSI	OH - BaP	0.66	0.04
LSI	UGT	-0.60	0.03
CYP1a	MT mRNA	- 0.63	0.02
CYP1a	UGT	0.60	0.03
Vtg	EROD	-0.65	0.04
HO-1	MT mRNA	0.57	0.04

Spearman's rank correlation results for males from the outer Oslofjord; variables, Rs: Spearman`s rank correlation coefficient and p– value.

Variable	Variable	Rs	P
Weight	Length	0.90	<.0001
Length	OH - Phen	-0.55	0.03
OH - Phen	Vtg	-0.66	0.04
OH - Phen	HSP70	0.83	0.002
OH - Phen	CYP1a	-0.72	0.01
CYP1a	HSP70	-0.61	0.05
Vtg	ALA-D	-0.77	0.009
UGT	GSI	-0.64	0.04

Chemical list and solutions

List of chemicals used in this thesis

Chemical	Producer no	Producer
Acetic acid	33209	Sigma-Aldrich
Acetylthiocholine iodid	A5751	Sigma-aldrich
Albumine fra bovine serum	A7906	Sigma-aldrich
Alkaline cupper Tartrate	500-0113	BioRad
Ammonium Chloride	1.01145	MERCK
Ammonium hydroxide	K34656632 519	MERCK
Bovine Serum Albumin	A2153	Sigma Aldrich
Dimethylsulfoxide	472310	Sigma-Aldrich
Dinatriumhydrogenphosphat	1.0658	MERCK
Dipotassium hydrogene triphosphate	A140504 924	MERCK
Dipotassium triphosphate	22,131-7	Sigma Aldrich
Dithiobisnitrobenzo acid	D8130	Sigma-aldrich
Dithiothreitol	MB1015	Melford lab
Ethylenediaminetetraacetic acid	ED2SS	Sigma-aldrich
Etylenediamintetraacid disodium	E7889	Sigma- Aldrich
Folin reagent	500-0114	BioRad
Hexaamminecobalt(III) chloride	H-7891	Sigma-Aldrich
Mercury(II) chloride	203777	Sigma-Aldrich
Metallothionin I&II from rabbit	M 7641	Sigma-aldrich
Perchloric acid 70%	CODE article: 20589.293	PROLABO
Porphobilinoge	P-1134	Sigma-aldrich
Resorufinethyl	E 3763	Sigma-aldrich
Resorufin sodium salt	R 3257	Sigma-aldrich
Trichloric acid	1.00807	MERCK
Triton® X-100	T8787	Sigma-aldrich
Trizma®base	T-1503	Sigma-aldrich
β-glucoronidase/arylsulfatase	1.04114	MERCK
δ-aminolevulinsyre hydrogen chlorid	A3785	Sigma-Aldrich
ρ-dimetylaminobenzaldehyd (Ehrlich`s reagens)	D2004	Sigma-Aldrich

Solutions used biomarkers

EROD	
Buffer	0.1M potassium- phosphate, pH 8.0 (from K2HPO4 and KH2PO4 in distillated water)
NADPH	50 mM in buffer
7-etoksyresorufin	0.2 mM in DMSO
Standard	1mM resorufin in DMSO

ALA-D	
Dilution buffer	
Sodiumphosphate pH 7.0	100ml 0.1M
Triton X-100	1ml
dH20	To a total of 200 ml
Modified Ehrlich reagens	
HgCl	0.35g in 6ml dH2O
70% perchloricacid	20ml
Acetic acid	to a total of 110ml
Ehrlich reagent	2 gram
Percipitation buffer	
Trichlorid acetic acid	40.0 g/l
HgCl	27.0 g/l
ALA-reagens	
δ-aminolevulin acid	33.5 mg
Dilution buffer	50 ml

MT	
Electrolyte	
Co(NH3)6Cl3	0.321 g/l
NH4Cl	3.4 g/l
NH4OH	154 ml up to 1 L

AChE	
Photassiumphosphate buffer	0.1M, with PH 7,2
AChE reagens (AChE)	
0.1M Photassiumphosphate buffer	30 ml
0.075M ATC	0.2ml
DTNB	1.0ml

RT-qPCR

Settings Lightcycler

Step	Temperature (°C)	Acqusition mode	Hold	Ramp rate (°C/s)	Acquiaitions
Pre-incubation	95.0	None	00:05:00	4.4	-
Amplification	95.0	None	00:00:10	4.4	-
Primer dependent		Single	00:00:20	4.4	
Melting curve	95.0	None	00:00:05	4.4	-
	65.0	None	00:01:00	2.2	-
	97.0	Continuous	-	-	5 - 10
Cooling	4.0	None	00:00:10	1.5	-

Bioanalyzer

All reagents were kept at 4 °C (the RNA ladder was kept at -80 °C and thawed immediately before use). Because of light sensitivity, the dye and the dye mix were covered with aluminium foil. RNA 6000 nanogel matrix (550 µl) was added to a spin filter and spun for 10 min at 1500 x g before aliquotting 65 µl of the matrix into 0.5ml RNase-free microfuge tubes and stored at 4 °C. The dye was spun down for 10 sec and 1 µl was added to a 65 µl tube of gel matrix. The tube with gel matrix and dye was vortexed thoroughly for 10 seconds and centrifuged at maximum speed for 10 min. Before using the Bioanalyzer, the electrodes were cleaned with electrode cleaners first filled with iso - propanol, then RNase free water. All samples, as well as the RNA ladder, were denaturated by heating at 70 °C on a heating block for 2 minutes, then chilled on ice for 5 min before briefly centrifuged. This denaturation step is to avoid the RNA from making secondary structures which can influence the speed at to which the molecules passes through the gel. The chip was placed on the priming station and loaded with 9 µl of the gel-dye matrix according to the protocol. In addition to 1 µl of ladder and 1 µl of RNA isolate, 5 µl of marker was added to the chip to align the samples. Before inserted into the machine, the chip was vortexed for 1 min at 2400 rpm in the adapter.